THE BIOCHEMICAL JOURNAL

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Preparation of Hydrophilic Colloids of Brain Kephalins

By Y. KIMURA and Y. NAGAI

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(*Received* 12 *August* 1959)

Phospholipids can be extracted from certain plant materials as a completely clear aqueous solution (Thierfelder & Klenk, 1930). However, phospholipids usually give a stable translucent emulsion with water. We have found that translucent, water emulsions of inositol phosphatide fraction (Folch's fraction I) or phosphatidylserine (Folch's fraction III) from brain tissue (Folch, 1942) quickly become transparent if ethylenediaminetetra-acetate is added at pH 10. The clear solution is stable even after removal of the reagents by dialysis.

MATERIALS AND METHODS

Brain-kephalin fractions. Phosphoinositide fraction (fraction I), phosphatidylserine (fraction III) and phosphatidylethanolamine (fraction V) were prepared from ox brains by the method of Folch (1942). Fraction I, before dialysis, contained 5·36% of P, 1·18% of N (P/N, 2·05) and 21·4% of ash. Fraction III, before dialysis, contained 3·54% of P, 1·55% of N (P/N, 1·03) and 14·3% of ash. After dialysis for 9 days it contained 3·55% of P, 1·50% of N (P/N, 1·07) and 1·44% of carboxyl N (carboxyl N/N, 0·96). Fraction V, before dialysis, contained 3·42% of P, 2·01% of N (P/N, 0·78) and 5·2% of ash. Each lipid sample was examined by paper chromatography in phenol–water (85:15, v/v) by which phosphatidylserine and phosphatidylethanolamine could be separated (Amelung & Böhm, 1954). Non-dialysed samples of fraction I gave a single elongated spot of lipid accompanied by several amino acid spots which were absent from the dialysed sample. Fractions III and V gave only single lipid spots with R_F values of 0·62 and 0·82 respectively.

The lipids were hydrolysed by heating 10–20 mg. with 5 ml. of 6N-HCl in a sealed tube for 20–40 hr. at 105°. The hydrolysate, after removal of excess of HCl, was submitted to one- and two-dimensional chromatography with phenol–water (80:20, v/v), or butanol–acetic acid–water (40:10:10, by vol.) or both of these solvents. In the hydrolysate of the dialysed sample of fraction I, serine and ethanolamine were identified as well as three ninhydrin-reacting spots with the R_F values of aspartic acid, glutamic acid and alanine. Fractions III and V each gave single ninhydrin-positive spots which were identified as serine and ethanolamine respectively. Inositol (Nagai & Kimura, 1958) was detected in fraction I but not in fractions III and V. Choline (Hawke & Lea, 1953) was absent from all three fractions.

Brain-'lecithin' fraction. An ethanol-soluble, acetone-insoluble lipid preparation from ox brain was used as a lecithin-rich fraction.

Reagents for clearing lipid emulsions. Aq. NH_3 soln.–NH_4Cl stock buffer solution (reagent A) was 70 g. of NH_4Cl and 570 ml. of conc. aqueous NH_3 (sp.gr. 0·90) diluted with water to 1 l. Clearing agent at pH 10 (reagent B) was 5 ml. of reagent A added to 50 ml. of aqueous 5% (w/v) solution of ethylenediaminetetra-acetate (EDTA; A.R. disodium dihydrate salt). EDTA solution at pH 10 for dialysis (reagent C) was 1 ml. of reagent A added to 50 ml. of aqueous 0·5% solution of EDTA.

RESULTS

Preparation of a transparent aqueous solution of kephalins. Reagent B was added in portions, with stirring, to a fresh, non-dialysed preparation of fraction I or III or to the conc. aqueous suspensions until the final concentration of lipid was about 3% (w/v). The mixtures soon became clear, as shown in Fig. 1. EDTA and other diffusible substances could be removed from the clear solution by dialysis through cellophan at 4° with constant stirring against 100 vol. of reagent C for 24 hr., then against 100 vol. of diluted reagent A (1:50, v/v; pH 10) for 24 hr. and finally for a total of 5 days against nine changes of 100 vol. of distilled water. A stable clear solution could also be obtained from fraction III simply by dialysing against water immediately after clearing by reagent B.

The pH values of the dialysed transparent solutions were about 3. The pH of the original water-emulsion was about 6·4 in fraction I and 4·6 in fraction III. The dialysed solutions contained much less ash, namely, 1·3% in fraction I and 3·0% in fraction III. The lipid samples which have been recovered by freeze-drying from the dialysed clear solution were submitted to infrared spectral analysis. The characteristic absorptions of EDTA (1630 cm.$^{-1}$, 1400 cm.$^{-1}$, 1367 cm.$^{-1}$, 1317 cm.$^{-1}$, 1290 cm.$^{-1}$ and five bands between 960 cm.$^{-1}$ and 800 cm.$^{-1}$) were absent. The spectra were closely similar to that of the metal-free kephalins, which are given elsewhere (Kimura & Nagai, 1960).

Stability of the clear solution. The hydrophilic colloids thus obtained were stable under ordinary conditions, but on freezing and thawing the transparent solution from fraction III changed into a gross suspension and that from fraction I into a slightly translucent emulsion. Addition of EDTA

and of ammonia at a suitable pH restored the transparency of the suspension from fraction III, but the emulsion from fraction I remained turbid. Even with fraction III, however, several repetitions of the same treatment, freezing, thawing and clearing, brought about an irreversible change into suspension. This seemed to be reflected in the behaviour of the lipid. Dialysed fraction I gave a clear solution by the EDTA treatment in the same way as the undialysed material, but the resulting solution changed irreversibly into a turbid emulsion if it was dialysed to remove EDTA. This turbidity caused by removal of EDTA was not observed with fraction III.

The clear aqueous lipid solutions remained transparent on the addition of alkali but immediately become turbid on the addition of neutral salts such as sodium, potassium or ammonium chloride. The turbid solution could be cleared again by adding EDTA and alkali. Shaking with chloroform and ether also caused turbidity. Lipid preparations recovered from the clear aqueous solutions by freeze-drying showed no marked change in solubility behaviour. They were easily soluble in chloroform and formed translucent or opaque emulsions with water.

Clearing action of ammonia. In the absence of EDTA, aqueous ammonia had a clearing action but only when sufficient ammonia was added to the aqueous emulsion of lipids to bring the pH to 10·8. The resulting clear solution was far less stable than that obtained by the use of EDTA. The clear aqueous solution of fraction III remained so after removal of ammonia, but the turbidity caused by adding neutral salts or by freezing and thawing did not disappear permanently on the addition of ammonia, even if transient clarification occurred. When the preparation of lipid had been purified by dialysis against water for 9 days, the clear aqueous solution, obtained by adding ammonia, became

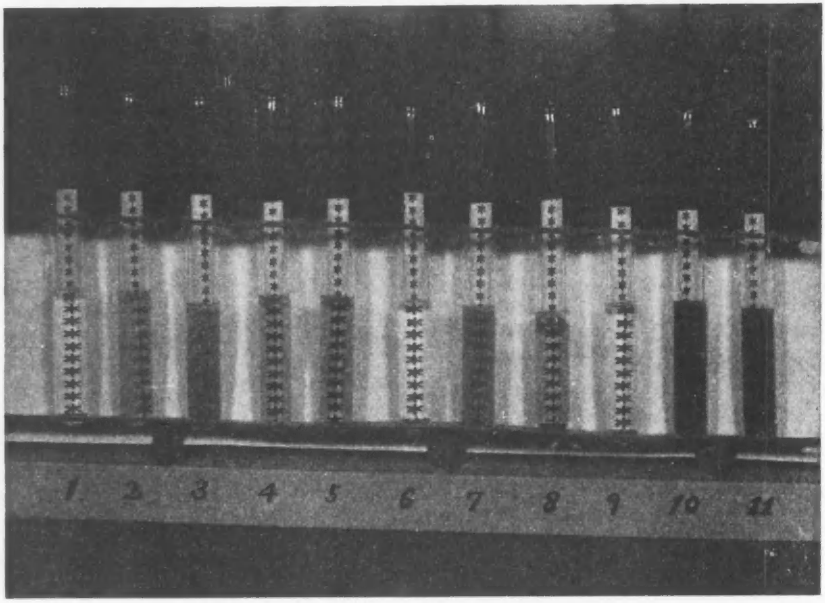

Fig. 1. Clearing effect of EDTA on aqueous emulsions of brain kephalins. 1, Plain water; 2, water emulsion of fraction I (30 mg. of lipid/ml.); 3, Aq. NH_3 soln.–NH_4Cl buffer (pH 10) emulsion of fraction I (30 mg. of lipid/ml.); 4, transparent solution obtained by adding EDTA to the buffered emulsion (3); 5, unchanged transparent solution obtained after dialysis of the solution (4) against water; 6, plain buffer solution of aq. NH_3 soln.–NH_4Cl (pH 10); 7, aq. NH_3 soln.–NH_4Cl buffer (pH 10) emulsion of fraction III (20 mg. of lipid/ml.); 8, transparent solution obtained by adding EDTA to the buffered emulsion (7); 9, unchanged transparent solution obtained after dialysis of the solution (8) against water (8 mg. of lipid/ml.); 10, aq. NH_3 soln.–NH_4Cl buffer (pH 10) emulsion of fraction V (30 mg. of lipid/ml.); 11, buffered emulsion (10) with addition of EDTA showing no clearing. (Test tubes are approx. 5 mm. in diameter.)

turbid again on removing the ammonia by dialysis. A translucent emulsion of fraction I also becomes transparent when ammonia was added, but it soon became very turbid again, and on standing a white precipitate formed with a clear supernatant which became turbid on dialysis. The white precipitate contained inorganic phosphate and lipids.

SUMMARY

1. When a turbid water emulsion of inositol phosphatide fraction (Folch's fraction I) or phosphatidylserine (Folch's fraction III) from brain tissues is added to a solution of ethylenediaminetetra-acetate at pH 10, the solution quickly becomes transparent and remains so even after removal by dialysis of the reagents added. Such a phenomenon is not seen with phosphatidylethanolamine (Folch's fraction V) or with a brain-'lecithin' fraction.

2. The clear solutions become turbid on freezing and thawing, and on the addition of chloroform, ether or neutral salts.

3. Aqueous ammonia also has a clearing action at higher pH values, but the resulting clear solutions were far less stable than those obtained by the use of ethylenediaminetetra-acetate.

We wish to express our indebtedness to Mrs R. Irie and Miss M. Iwanaga, of the Division of Chemistry in this Institute, for the elementary analyses. This work was supported by a Grant for Fundamental Research obtained from the Ministry of Education.

REFERENCES

Amelung, D. & Böhm, P (1954). *Hoppe-Seyl. Z.* **298**, 199.
Folch, J. (1942). *J. biol. Chem.* **146**, 35.
Hawke, J. C. & Lea, C. H. (1953). *Biochem. J.* **54**, 479.
Kimura, Y. & Nagai, Y. (1960). *Biochem. J.* **77**, 3.
Nagai, Y. & Kimura, Y. (1958). *Nature, Lond.*, **181**, 1730.
Thierfelder, H. & Klenk, E. (1930). *Die Chemie der Cerebroside und Phosphatide*, pp. 177–8. Berlin: Springer Verlag.

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Infrared Spectra of Brain Phosphatidylserine

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(*Received* 19 *October* 1959)

Infrared spectroscopy has been applied to many complex lipids, but the infrared spectrum of phosphatidylserine seems not to have been published. Marinetti, Erbland & Stotz (1958) mention that a sodium salt of brain phosphatidylserine has more absorption at 6·1 μ and less at 2·9 μ than has the free acid form.

MATERIALS AND METHODS

Phosphatidylserine. This was prepared as described by Kimura & Nagai (1960).

Metal-free phosphatidylserine. This was prepared according to the procedure of Folch (1948).

Monosodium phosphatidylserine. In this the atomic ratio Na:P was 1:1; it was prepared by the addition, with cooling and stirring, of the calculated amount of CO_2-free NaOH (0·01 N) to a suspension (2%, w/v) of the metal-free phosphatidylserine in CO_2-free water. The solution was then freeze-dried.

Disodium phosphatidylserine. In this the atomic ratio Na:P was 2:1 and it was prepared similarly.

Infrared spectroscopy. All infrared spectra were obtained by the KBr-disk technique. A Hitachi Model EPI-2 double-beam automatic-recording spectrophotometer (Hitachi Ltd., Tokyo, Japan) with an NaCl prism was used. The same infrared spectral patterns were obtained in a KBr disk, in chloroform solution and in Nujol film. When phosphatidylserine was dialysed against water for 9 days, its ash content was decreased and, in parallel with this, a similar spectral pattern to that of the metal-free form was obtained.

RESULTS AND DISCUSSION

The infrared spectra of the four forms of phosphatidylserine are shown in Fig. 1. All forms possess the following bands which can be correlated with specific molecular groups: 1750–1740 cm.$^{-1}$, C=O ester, stretching; 1475 cm.$^{-1}$ and 1375 cm.$^{-1}$, CH deformation; 1235 cm.$^{-1}$, P=O free, stretching; and 1050 cm.$^{-1}$, P—O—C stretching. Four absorption bands (1655 cm.$^{-1}$, 1525 cm.$^{-1}$, 1420 cm.$^{-1}$ and 1175 cm.$^{-1}$) are affected by the form of the material and thus must be attributed to the acidic (—CO_2H, ‖ —P—OH) and basic (—NH_2) groups of phosphatidylserine.

The 1655 cm.$^{-1}$ absorption, a characteristic of the original phosphatidylserine, vanishes in the metal-free form but reappears in the mono- and di-sodium forms, although the bands become broader

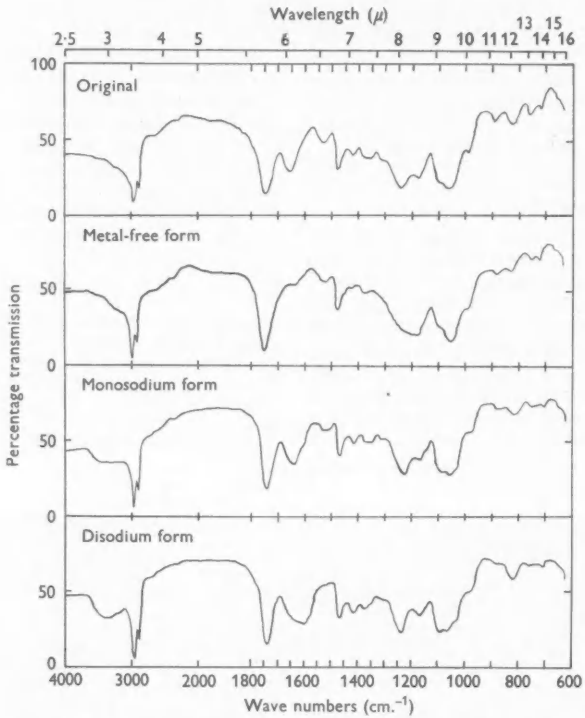

Fig. 1. Infrared-absorption spectra (KBr) of four different forms of phosphatidylserine.

and the absorption maxima shift to lower frequencies of 1645 and 1600 cm.$^{-1}$ respectively. The 1655 cm.$^{-1}$ band is always accompanied by a weaker band at 1420 cm.$^{-1}$ and the two bands are therefore assumed to be the symmetrical and anti-symmetrical vibrations of the ionized carboxyl group (Colthup, 1950; Fuson, Josien & Powell, 1952; Sutherland, 1952; Koegel, McCallum, Greenstein, Winitz & Birnbaum, 1957; Bellamy, 1958). Presumably the un-ionized carboxyl group absorbs at 1750 cm.$^{-1}$, since this band is more intense in the metal-free form.

In the original and monosodium forms, the band of the ionized carboxyl group is outside the range of 1610–1550 cm.$^{-1}$ which it occupies in simpler carboxylic and amino acids (Colthup, 1950; Fuson *et al.* 1952; Koegel *et al.* 1957; Bellamy, 1958). The reason for this is not known, but it is not surprising in view of the more complex structure of phosphatidylserine.

SUMMARY

1. Infrared spectra are reported for four forms of phosphatidylserine, namely the original preparations, metal-free, mono- and di-sodium derivatives, the last two denoting that the atomic ratios Na:P in the molecules are 1:1 and 2:1 respectively.

The authors wish to express their gratitude to Dr H. Watanabe, Department of Chemistry, for his helpful advice. The authors are also very much indebted to Professor T. Yamakawa, Department of Chemistry, for his useful advice.

REFERENCES

Bellamy, L. J. (1958). *The Infrared Spectra of Complex Molecules*, 2nd ed., pp. 174–5, 240–2. London: Methuen and Co. Ltd.

Colthup, N. B. (1950). *J. opt. Soc. Amer.* **40**, 397.

Folch, J. (1948). *J. biol. Chem.* **174**, 439.

Fuson, N., Josien, M. & Powell, R. L. (1952). *J. Amer. chem. Soc.* **74**, 1.

Kimura, Y. & Nagai, Y. (1960). *Biochem. J.* **77**, 1.

Koegel, R. J., McCallum, R. A., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957). *Ann. N.Y. Acad. Sci.* **69**, 94.

Marinetti, G. V., Erbland, J. & Stotz, E. (1958). *Biochim. biophys. Acta*, **30**, 41.

Sutherland, G. B. B. M. (1952). *Advanc. Protein Chem.* **7**, 291.

The Enzymic Composition of Nuclei Isolated from Radio-Sensitive and Non-Sensitive Tissues with Special Reference to Catalase Activity

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(*Received* 12 *October* 1959)

The many attempts to demonstrate early activation or inactivation of specific enzymes as a consequence of exposure to ionizing radiation *in vivo* have not succeeded in disclosing the biochemical origin of the radiobiological lesion. This failure may be due to localization of the primary biochemical events at certain sites within the cell, for in such circumstances the earliest changes might escape detection in studies on whole tissues or homogenates. Such localized intracellular responses have been observed in the behaviour of mitochondrial deoxyribonuclease II (Goutier-Pirotte & Thonnard, 1956) and in the uptake of ^{32}P into liver ribonucleic acid (Payne, Kelly & Entenman, 1952). It seemed likely that in view of the marked disturbance of nuclear function after irradiation, a comparison of the enzymic complement of the nucleus in radio-sensitive (i.e. those tissues which show early histological deterioration after exposure to sublethal radiation) and non-sensitive tissues might provide a clue to the nature of the primary biochemical lesion.

About thirty nuclear enzymes are known, but unfortunately many of these have been described in tissues from slaughter-house animals which are not suitable for radiobiological studies. The rat, which is very satisfactory for such purposes, has been used for the present work. A number of nuclear enzymes have been assayed and their activities expressed on the basis of deoxyribonucleic acid phosphorus content as recommended by Gray & Deluca (1956).

EXPERIMENTAL

Enzyme assays

Adenosine deaminase. The method depended upon the change of $7{\cdot}2 \times 10^3$ in the molar extinction coefficient at 260 mμ which accompanies the conversion of adenosine into inosine (Beaven, Holiday & Johnson, 1955). The test system contained 0·12 mM-adenosine, 12 mM-phosphate buffer, pH 7·4, and 0·5 ml. of tissue suspension in a total volume of 3 ml.; the tubes were incubated for 40 min. at 37° and the reaction was stopped by adding 1 ml. of 2M-$HClO_4$. After centrifuging, samples (1 ml.) were diluted to 3 ml. and their extinctions measured. The activity was expressed as μmoles of adenosine deaminated/min./mg. of deoxyribonucleic acid phosphorus (DNA-P).

Adenosine triphosphatase. The procedure of van Bekkum (1955) was used. Incubations were carried out at both 0° and 37° and the inorganic orthophosphate (*Pi*) which was released was measured by the method of Berenblum & Chain (1938). The activity was expressed as μg. of Pi released/min./mg. of DNA-P.

Alcohol dehydrogenase. This enzyme, prepared as described by Racker (1950) from yeast, was used for the assay (Kornberg, 1950) of diphosphopyridine nucleotide (DPN).

Ascorbic acid oxidase. The enzyme was assayed by estimating the rate of disappearance of ascorbic acid as measured by the reduction of 2:6-dichlorophenolindophenol (*Methods of Vitamin Assay*, 1951). The incubation system consisted of 1 ml. of 0·2M-acetate buffer, pH 5·0, 1 ml. of ascorbic acid (17·5 μg./ml.) and 1 ml. of tissue suspension. After incubation at 37° the reaction was stopped by adding 1 ml. of 2M-$HClO_4$ and the tubes were centrifuged. Portions of the supernatant fluids (2 ml.) were pipetted into spectrophotometer cells, followed by 2 ml. of 2:6-dichlorophenolindophenol (16 μg./ml.). Readings of extinction at 520 mμ were taken at 5, 15 and 30 sec. so that by extrapolating to zero time the decrease in extinction due to ascorbic acid alone could be determined. The activity was expressed as μg. of ascorbic acid oxidized/min./mg. of DNA-P.

Catalase. The procedure of Dale & Russel (1956) was modified to give a colour which was less markedly temperature-dependent. Stock titanium reagent was prepared by boiling 20 ml. of commercial titanous chloride solution (British Drug Houses Ltd., 15%) with 10 ml. of 5N-H_2SO_4 and adding a few drops of conc. HNO_3 to just discharge the pink colour. The yellow solution was then made up to 50 ml. with water to give the stock reagent, which was diluted 1:10 for use. 0·05M-Hydrogen peroxide (1 ml.) and 1 ml. of 0·02M-phosphate buffer, pH 7·0, were pipetted into each centrifuge tube. The reaction was begun by rapid addition of 1 ml. of tissue and stopped by blowing in 2 ml. of 5N-H_2SO_4, incubation being carried out at 0°. Acid was added before the tissue to obtain the zero time samples. The acidified suspensions were then centrifuged and samples (1 ml.) transferred to 10 ml. graduated tubes. To each of these was added 1 ml. of diluted titanium reagent and then water to the 10 ml. mark. The tubes were left standing for 15 min. at room temperature and extinctions were determined at 407 mμ. The activity was expressed as μmoles of H_2O_2 decomposed/min./mg. of DNA-P.

Cytosine and cytidine deaminases. The procedure was that described for adenosine deaminase except that 0·12 mM-cytosine and 0·12 mM-cytidine were substituted

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for adenosine. Extinctions were measured at 280 mμ, at which wavelength a 58% decrease in absorption accompanies the deamination (Hotchkiss, 1948). The activity was expressed as μmoles of cytosine or cytidine deaminated/min./mg. of DNA-P.

Diphosphopyridine nucleotide (DPN) nucleosidase and diphosphopyridine nucleotide pyrophosphatase. These enzymes were assayed together with an incubation medium consisting of 1·2 mM-DPN, 0·06 M-phosphate buffer (pH 7·1) and 0·2 ml. of tissue suspension in a total volume of 0·5 ml.; initial and final samples were obtained by heating the tubes for 1·5 min. at 100° before and after incubation at 37° for 15 min. Samples (0·2 ml.) were pipetted into 3 ml. of M-NaCN for DPN-nucleosidase activity (Zatman, Kaplan & Colowick, 1953) and into 3 ml. of the alcohol-dehydrogenase system for estimations of DPN-pyrophosphatase activity (Kornberg & Pricer, 1950). Molar extinction coefficients of $6{\cdot}3 \times 10^3$ at 325 mμ (Colowick, Kaplan & Ciotti, 1951) and $6{\cdot}22 \times 10^3$ at 340 mμ (Horecker & Kornberg, 1948) were used for the conversion of DPN into the cyanide derivative and into the reduced form respectively. Correction of the total change in DPN content, given by the alcohol-dehydrogenase method for the DPN cloven at the pyridinium linkage, gave the amount decomposed by pyrophosphatase action. The activity was expressed as μmoles of DPN decomposed/hr./mg. of DNA-P.

Diphosphopyridine nucleotide pyrophosphorylase. This enzyme was estimated by the increase in the rate of disappearance of enzymically active DPN which occurs in the presence of inorganic pyrophosphate (Kornberg, 1950). The activity was expressed as μmoles of DPN decomposed/hr./mg. of DNA-P.

Diphosphopyridine nucleotide synthesis by the desamido pathway. Preiss & Handler (1957) have described a system in human erythrocytes which forms DPN from nicotinic acid with the desamido analogues of nicotinamide mononucleotide and DPN as intermediates. Nuclear preparations were tested for activity in the following medium: 4 mM-nicotinic acid, 8 mM-phosphate buffer (pH 7·4), 4 mM-adenosine triphosphate (ATP, K salt), 8 mM-glutamine, 80 mM-nicotinamide (to inhibit DPN nucleosidase) and 0·5 ml. of tissue to give a final volume of 2·5 ml. As a source of the ribose moiety of DPN, glucose, ribose 5-phosphate and 5-phosphoribosyl pyrophosphate have been used at concentrations of about 4 mM. Tubes were incubated at 37° for periods of up to 180 min. and the reaction was stopped by heating them for 1·5 min. at 100°. After rapid cooling in ice the contents were centrifuged and the supernatant fluids assayed for DPN by the alcohol-dehydrogenase method. The activity was expressed as μmoles of DPN synthesized/hr./mg. of DNA-P.

Inorganic pyrophosphatase. The method employed was that of Heppel (1955). The activity was expressed as μg. of Pi liberated/min./mg. of DNA-P.

5′-Nucleotidase. The assay system was that of Heppel & Hilmoe (1955). The activity was expressed as μg. of Pi liberated/min./mg. of DNA-P.

Phosphatases. Acid and alkaline phosphatases were determined by a modification of the method of Huggins & Talalay (1945). A more dilute alkaline glycine reagent was found to give a more stable colour. This solution consisted of 1·38 g. of glycine, 1·5 g. of NaOH and 6·0 g. of $Na_4P_2O_7, 10H_2O$ in 1 l. To avoid the gelation which occurs when nuclei are exposed to alkaline solutions, the reaction was stopped with ethanol. The medium for acid phosphatase contained 0·1 M-acetate buffer, pH 5·5, and the medium for the alkaline enzyme contained M-veronal buffer, pH 7·4, and 0·04% (w/v) of $MgCl_2, 2H_2O$. Both media also contained mM-phenolphthalein phosphate (L. Light and Co. Ltd.). Samples of either acid or alkaline medium (2 ml.) were incubated at 37° with 0·5 ml. of tissue suspension. The reaction was stopped by adding 2 ml. of ethanol and centrifuging. Samples (2 ml.) of the supernatant fluids were treated with 2 ml. of alkaline glycine reagent and the extinctions measured at 555 mμ. The activity was expressed as μg. of phenolphthalein released/hr./mg. of DNA-P.

Peroxidase. The assay described by George (1953) was used. The activity was expressed as μmoles of H_2O_2/min./mg. of DNA-P.

Polynucleotide phosphorylase. This enzyme was estimated by measuring the rate of exchange of inorganic [^{32}P]orthophosphate with the terminal phosphate group of adenosine diphosphate (ADP) in the system described by Hilmoe & Heppel (1957). The activity was expressed as μg. of Pi exchanged with ADP/hr./mg. of DNA-P.

Ribonuclease. This enzyme was assayed by determining the release of acid-soluble nucleotides as u.v.-absorbing material. The incubation medium contained 0·5 ml. of yeast ribonucleic acid (RNA; 1%), 0·5 ml. of buffer (0·1 M acetate, pH 5·5, or 0·1 M-2-amino-2-hydroxymethyl-propane-1:3-diol–HCl buffer, pH 8·0), 0·8 ml. of water and 0·2 ml. of tissue. The reaction was stopped by the addition of 2 ml. of acid ethanol (Roth & Milstein, 1952) and, after centrifuging, the extinctions of the supernatant fluids were read at 260 mμ. Activities were calculated from a value for $E_{260\ m\mu}$ of 393 for a solution of RNA containing 1 mg. of phosphorus/ml., derived from the data of Volkin & Carter (1951). The activity was expressed as mg. of RNA phosphorus (RNA-P) released/hr./mg. of DNA-P.

Xanthine oxidase. The method was based on that described by Kalckar (1947) but the presence of uricase in our spleen-nuclear preparations made it necessary to modify the incubation medium. Both uricase and xanthine oxidase are inhibited by cyanide, but if the substrate is added before the cyanide, xanthine oxidase retains its activity (Szent-Györgyi, 1926). Therefore NaCN was added finally to all test systems at a mM concentration. The activity was expressed as μmoles of uric acid formed/min./mg. of DNA-P.

Materials and methods

Animals. Male and female animals (wt. 80–120 g.) of the laboratory strain of Wistar rats were used. They were decapitated and the organs were rapidly removed and placed in ice-cold medium. In experiments on the catalase activity of pigeon liver, adult male birds were killed by decapitation.

Irradiation. X-irradiation was carried out *in vivo* at the M.R.C. Radiobiological Research Unit, Harwell. The characteristics of the radiation were: half-value layer 1·2 mm. Cu, peak voltage 250 kv at 14 mA. Irradiation *in vitro* was effected by means of two 250 mC radium sources. The dose rates were 67 r./min. total body radiation *in vivo* and either 2·8 or 43·6 r./min. *in vitro* as measured by the oxidation of $FeSO_4$ according to the method of Miller (1950).

Preparation of nuclear suspensions. Three media were used: medium A was 0·25 M-sucrose, 3·3 mM-$CaCl_2$, 5 mM-triethanolamine–HCl adjusted with NaOH to pH 6·8; medium B was as A, but with 1·8 mM-$CaCl_2$; medium C was 45 mM-glucose, 85 mM-KCl, 8·5 mM-NaCl, 2·5 mM-$CaCl_2$, 2·5 mM-$MgCl_2$ and 5 mM-triethanolamine–HCl adjusted to pH 6·8. An account of the procedures used for the isolation of cell nuclei has appeared previously (Creasey & Stocken, 1959). For the present studies lymph nodes were obtained exclusively from the cervical region of the animals, except in one experiment when ileac nodes were used. Nuclei were isolated from pigeon liver in exactly the same way as from the corresponding rat tissue.

Deoxyribonucleic acid estimation. Burton's (1956) modification of Dische's (1930) diphenylamine method was used. Results were expressed as DNA-P.

Chemicals. The reagents were of AnalaR grade where available. ATP was prepared from rabbit muscle according to Needham (1942). 2-Amino-2-hydroxymethylpropane-1:3-diol was obtained from the Sigma Chemical Co. as 'Sigma 7–9' and was purified by recrystallization from hot aqueous 90% ethanol. Triethanolamine–HCl was obtained by threefold recrystallization from aqueous ethanol of the product prepared by neutralization of commercial-grade triethanolamine with HCl. RNA with a low acid-soluble blank was extracted from baker's yeast as described by DiCarlo & Schultz (1949). Ribose 5-phosphate was prepared by the acid hydrolysis of adenosine 5′-monophosphate (LePage & Umbreit, 1943). 5′-Phosphoribosyl 1-pyrophosphate was obtained by the action of an enzyme from pigeon liver upon ribose 5′-phosphate and ATP (Kornberg, Lieberman & Simms, 1955).

RESULTS

Nuclei were isolated from lymph node, kidney, spleen and thymus gland in medium A and from brain and pigeon and rat liver in medium B. Certain enzymes could not be detected in any of the preparations tested and these included ascorbic acid oxidase, cytosine and cytidine deaminase, peroxidase and the desamido pathway of DPN synthesis. In these cases liver, spleen and thymus nuclei were tested. The results of the other assays appear in Tables 1–3. Liver-nuclear preparations were generally more active than those from the thymus, especially with respect to their catalase and phosphatase activities. Adenosine deaminase and xanthine oxidase activities were, however, similar in all the tissues which were examined. Polynucleotide-phosphorylase activity was very low in the liver nuclei so that it is unlikely that its absence from thymus constitutes a significant distinction between these tissues. The activity of liver nuclei in the metabolism of pyridine nucleotides was found to be greater than that of thymus-nuclear preparations.

The most notable feature observed was the high level of catalase in the non-sensitive tissue nuclei (Table 3). Such a marked difference between the two types of tissue nuclei made it seem not unlikely that there might exist some correlation between enzyme distribution and radio-sensitivity. It was of interest therefore to see whether radiation would affect the activity of this enzyme. Rats were irradiated with varying doses of whole-body radiation and killed 1 hr. later. The catalase levels in tissues from these rats were compared with those from similar animals which had been treated in the same way except for the radiation. It was noted (Table 4) that the activity associated with the isolated nuclear fraction diminished with increasing doses of radiation. There appeared to be a slight

Table 1. *Activity of nuclear preparations from control rats in the metabolism of nucleotides*

All tissue fractions were isolated in sucrose media. Medium A was used for spleen and thymus glands and medium B for the liver. For the composition of media see the Materials and Methods section. The number of determinations together with the maximum range of the figures are shown in parentheses. For details of expressions used for enzyme activities see the Experimental section.

Enzyme		Spleen	Thymus gland	Liver
Adenosine deaminase (μmole/min./mg. of DNA-P)		0·17 (4) (0·15–0·21)	0·14 (2) (0·12–0·16)	0·23 (3) (0·19–0·26)
DPN nucleosidase (μmoles/hr./mg. of DNA-P)		—	7·57 (4) (6·56–8·37)	47·9 (2) (43·1–54·7)
DPN pyrophosphatase (μmoles/hr./mg. of DNA-P)		—	3·96 (3) (2·99–4·34)	17·5 (2) (15·7–19·2)
DPN pyrophosphorylase (μmoles/hr./mg. of DNA-P)		—	4·47 (2) (4·05–4·89)	43·6 (2) (36·8–50·4)
Polynucleotide phosphorylase (μg. of ^{32}Pi exchanged/hr./mg. of DNA-P)		—	Zero (2)	16·0 (2) (12·8–19·2)
Ribonuclease (mg. of RNA-P/hr./mg. of DNA-P)	pH 5·5	—	0·038 (3) (0·031–0·045)	0·407 (2) (0·362–0·451)
	pH 8·0	—	0·333 (3) (0·262–0·421)	0·962 (2) (0·807–1·017)
Xanthine oxidase (μmole/min./mg. of DNA-P)		0·068 (4) (0·046–0·100)	—	0·054 (3) (0·041–0·060)

Table 2. *Phosphatase activities of nuclear suspensions*

All tissue fractions, except where otherwise indicated, were isolated in sucrose medium A or B. The former was used for spleen and thymus gland; the latter for liver. The compositions of media A, B and C are given in the Materials and Methods section. The number of determinations together with the maximum range of the figures are shown in parentheses.

Enzyme		Spleen	Thymus gland	Liver
Acid phenolphthalein phosphatase (μg. of phenolphthalein/hr./mg. of DNA-P)		21·3 (5) (16·2–29·1)	0·60 (3) (0·43–0·76)	3·7 (4) (3·2–4·1)
Alkaline phenolphthalein phosphatase (μg. of phenolphthalein/hr./mg. of DNA-P)		2·21 (5) (1·83–2·58)	0·02 (4) (0·01–0·04)	0·44 (4) (0·37–0·52)
Inorganic pyrophosphatase (μg. of Pi/min./mg. of DNA-P)		—	3·37 (3) (2·98–3·61)	18·6 (3) (16·3–19·4)
Adenosine triphosphatase (μg. of Pi/min./mg. of DNA-P)	37°	—	44·2 (2) (40·3–48·1)	102·0 (2) (91·1–112·9)
	0°	—	2·86 (4) (2·26–3·51)	8·5 (3) (6·2–9·1)
	medium C 0°	—	2·83 (3) (2·52–3·20)	—
5′-Nucleotidase (μg. of Pi/min./mg. of DNA-P)		—	12·9 (2) (11·3–14·5)	545·0 (2) (468–622)

Table 3. *Catalase activities of various nuclear preparations*

All tissue fractions except liver and brain were prepared in medium A. For liver and brain medium B was used.

Tissue	Mean activity (μmoles of H_2O_2 decomposed/ min./mg. of DNA-P)	No. of experiments	Range of rates
Pigeon liver	3380	4	3120–3860
Rat liver	2320	5	2130–3150
Rat kidney	2920	3	2510–3530
Rat brain	590	3	535–630
Rat spleen	106·4	4	72·4–115·3
Rat thymus gland	16·3	7	11·8–22·4
Rat lymph node	37·1	5	29·8–44·6

Table 4. *Changes in catalase activities of tissues from rats which had been exposed to whole-body X-irradiation* 1 *hr. before*

All tissue fractions were prepared in medium A, except for liver where medium B was used. The catalase activities of tissue fractions from irradiated animals are grouped with those of tissues from the corresponding control rats, which, apart from the radiation, had been treated similarly.

	Catalase activities (μmoles of H_2O_2 decomposed/min./mg. of DNA-P)					
Tissue	Control	50 r.	Control	200 r.	Control	1000 r.
Thymus nuclei	15·0	13·3	11·8	7·8	16·4	9·0
	14·2	16·3	22·4	19·2	18·2	12·5
Lymph-node nuclei	42·6	32·8	29·8	22·1	40·8	23·3
	32·3	29·7	41·5	27·4	—	—
Liver nuclei	2740	2790	3140	2260	2860	2110
Thymus homogenate	64·2	71·3	58·9	63·1	80·6	83·8
	40·6	48·7	51·2	60·4	40·6	48·7
Lymph-node homogenate	153	187	169	179	174	188
	118	133	—	—	256*	307*

* Ileac lymph nodes were used; in all other cases the nodes were taken from the cervical region.

Table 5. *Removal of catalase from thymus-gland nuclei by the action of ribonuclease*

Nuclei were isolated from the thymus glands of control animals and of animals irradiated with 1000 r. 1 hr. before they were killed. The nuclear preparation was suspended in medium A. One portion was incubated at 0° for 40 min. with a protease-free solution of ribonuclease (L. Light and Co. Ltd.) at a final concentration of 0·05 mg./ml. and the remainder was treated similarly with medium alone. Activity is given as μmoles of H_2O_2 decomposed/min./mg. of DNA-P.

Tissue	Treatment received	Activity of sediment	Activity of supernatant	Total activity	Percentage of initial activity	RNA-P removed (μg./mg. of DNA-P)
Control	Medium	17·3	Zero	17·3	105	—
	Ribonuclease	7·3	7·4	14·7	90	9·76
Irradiated	Medium	8·6	Zero	8·6	96	—
	Ribonuclease	7·6	2·2	9·8	110	9·53

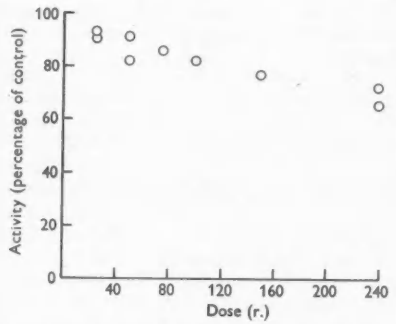

Fig. 1. Effect of γ-irradiation *in vitro* upon the catalase activity of nuclear preparations from the thymus gland of the rat. Activities are expressed as percentages of those of the unexposed portions of the preparations.

increase in the activity of the whole homogenate, but in view of the small changes obtained and lack of any correlation with the dose of radiation received this is of doubtful significance.

Although the results of the irradiation studies *in vivo* indicate that the nuclear-catalase activity is depressed, the rather wide range of the control values over the whole series of experiments somewhat obscures the significance of the data. In view of this, experiments with radiation *in vitro* were carried out. Nuclei were isolated from rat thymus glands in medium C and part of the suspension was exposed at 0° to γ-irradiation from a radium source. The catalase activity was then compared with that of the untreated portion thus eliminating the factor of control variation. The decrease in the activity of the irradiated samples (Fig. 1) not only serves to reinforce the findings *in vivo* but also indicates that the radiation is acting directly upon the nucleus and not upon some other part of the cell.

In an attempt to find other factors which might influence the level of catalase in cell nuclei, we studied the effect of ribonuclease action upon isolated nuclei. In these experiments both control and irradiated rats were used. The latter had received a total body radiation of 1000 r. 1 hr. before they were killed. Thymus nuclei were isolated in medium A and each preparation was divided into two portions. To one was added ribonuclease solution (0·05 mg./ml.) and to the other isolation medium alone. After 40 min. gentle shaking at 0° the tubes were centrifuged and the amounts of catalase in the sediment (i.e. the intact nuclei) and in the supernatant were determined. Whereas with medium alone no leakage of catalase could be detected, ribonuclease action released some of this enzyme as well as acid-soluble ultraviolet-absorbing material from the nuclei (Table 5). A similar experiment with deoxyribonuclease failed to show such an effect.

DISCUSSION

The literature concerning the enzymic composition of isolated cell nuclei has been adequately reviewed (Dounce, 1950*a*, *b*; 1952, 1954, 1955; Allfrey, Mirsky & Stern, 1955; Siebert & Smellie, 1957), and despite the problem of modification of nuclear composition during the standard isolation procedures (Stern & Mirsky, 1953; Dounce & Monty, 1955; Dounce, Witter, Monty, Pate & Cottone, 1955), a certain pattern of nuclear metabolic activity does emerge. Thus the nucleus is well equipped with enzymes for nucleotide metabolism, but appears to be lacking in oxidases, flavoproteins and cytochromes. In addition, liver nuclei are generally more active enzymically than those from most other tissues, a reflexion of the high metabolic activity of the whole organ. The data presented here conform to such a pattern, as for example in the activity of liver-nuclear preparations.

Stern & Timonen (1954) have suggested that ascorbic acid might function as an intermediate in nuclear oxidations and produced evidence of a correlation between mitotic activity and ascorbic acid content of certain plant tissues. The apparent absence of ascorbic acid oxidase from our preparations makes it unlikely that the acid can function in

this way in mammalian systems. However, the presence of DPN pyrophosphorylase in nuclear preparations from spleen (Potter & Grannis, 1953), liver (Hogeboom & Schneider, 1952) and thymus makes it most probable that DPN itself is available as a hydrogen acceptor. Reduced DPN could then be reoxidized in the mitochondria.

The distribution of the enzymes involved in DPN metabolism is of interest; liver nuclei are more active than both spleen (Potter & Grannis, 1953) and thymus preparations. Relatively low levels of DPN have been noted in tumour tissues (Glock & McLean, 1957) and Morton (1958) has suggested that the ability of a tissue to generate DPN in its cell nuclei may be the determining factor in the rate of growth. Tissues such as the liver are able to increase their capacity to synthesize DPN and thus to supply a large cytoplasm with this metabolite. In rapidly dividing tissues, the nuclei are unable to make this adjustment and are thus frequently subjected to a cytoplasmic stimulus (associated with the ATP level) which initiates the process of division. Although examination of a wider range of tissues and of the action of possible inhibitors of DPN pyrophosphorylase is advisable, and although the position with respect to the desamido pathway is not yet clear, the present data may certainly be reconciled with such a mechanism.

It does not appear that the metabolism of DPN is directly influenced by irradiation. In some preliminary experiments no change was detected in the activity of DPN nucleosidase, as well as of ribonuclease and adenosine deaminase, in thymus-nuclear suspensions 1–2 hr. after a total body radiation of 200 r. This is in agreement with the work of Eichel & Spirtes (1955), who were able to detect a decrease only in spleen DPNH after 980 r. when gross changes in cell population had occurred.

The only marked differentiation between radiosensitive and non-sensitive tissue nuclei which emerged from these studies was in their catalase activities. This enzyme has attracted some attention because of the possible mediation by peroxides in the development of the radiobiological lesion. Radiation of the purified enzyme has been shown to cause inactivation (Forssberg, 1947) and the work of Sutton (1956) has established that this effect was not due to the hydrogen peroxide produced by the action of ionizing radiation upon water but rather to free radicals interacting at the iron atom of the catalase molecule. Studies of the catalase levels *in vivo* have revealed no uniform change, both activation (Euler & Hevesy, 1942; Aronson, Fraser & Smith, 1956) and inactivation (Feinstein, Butler & Hendley, 1950; Mori, Momoki & Itô, 1951) having been reported. Certain of these observations may, at least in part, be attributed to other factors such as cell permeability (Aronson *et al.* 1956) or to changes in the rate of enzyme synthesis (Mori *et al.* 1951). However, some of the contradictions may spring from a possible dual role of the radiation. First, we have the radical-mediated inactivation and, secondly, there may be an increased availability of the enzyme, either by release through the cell membrane or by mobilization from intracellular sites, where it forms part of the particulate structure. Evidence pertaining to this latter possibility is seen in the work of Brown (1952), who found a more active catalase component which was associated with structural elements of the cell and was released by vigorous homogenization. Kaplan & Paik (1957) concluded from their studies of the action of u.v. light upon yeast-cell catalase that the enzyme was associated with ribonucleoprotein, and that radiation led to the dissociation of this complex. This gave rise initially to an increase in the apparent enzyme activity, and then, after a maximum had been reached, the activity fell off apparently through inactivation of the enzyme by radiation. The data on nuclear catalase presented here can be reconciled with the existence of some form of enzyme complex; this is suggested even by the fact that nuclei are able to retain a soluble enzyme such as catalase. As to the nature of the insoluble complex, the experiments with ribonuclease indicate that RNA may be involved. The results obtained after irradiation *in vivo* suggest that we are dealing with an effect which is possibly analogous to that observed by Kaplan & Paik, since there was a loss of the nuclear component without any decrease in the total cellular content. However, the experiments with irradiation *in vitro* seem rather to indicate that catalase is being inactivated. Attempts have been made to measure the amount of catalase which had leaked out of the nuclei during irradiation. With the usual assay system only traces of the enzyme were found, but in one experiment in which the hydrogen peroxide concentration had been lowered from 16·7 to 3·3 mM, a total initial activity of 18·4 units decreased to 15·7 units, of which 3·7 units were in the supernatant after irradiation (thymus nuclei; 240 r.). This suggests that more sensitive assay systems which did not subject the enzyme to substrate inactivation might disclose that the radiation had caused a leakage of the enzyme rather than an inactivation. The significance for the organism of this migration of catalase from its normal site of action is difficult to assess, but it is permissible to speculate. It has been known since the work of Keilin & Hartree (1945) that catalase can function in coupled oxidations, and it is probably in that capacity that the deficiency will be felt.

At the present time it seems probable that the biochemical mechanism whereby the radiobiological lesion is propagated and amplified involves a disruption of the ordered arrangement of enzyme systems, substrates and activating ions within the cell. Many such changes in intracellular distribution are already known, as, for example, the leakage of deoxyribonuclease II cited above, and the loss of nuclear sodium and potassium ions (Creasey, 1960), and it is likely that the consequences of such disorganization will be more far reaching than could possibly be the case if the damage were restricted to enzyme inactivation.

SUMMARY

1. A study has been made of the enzyme content of cell nuclei isolated from both radio-sensitive and non-sensitive tissues of the rat and from pigeon liver in an attempt to demonstrate a biochemical basis for differences in radio-sensitivity.

2. Catalase activity was found to be much greater in the nuclei from non-sensitive tissues.

3. Total body radiation led to a decrease in nuclear-catalase content, but no significant change in the activity of whole lymphatic tissues. The nuclear effect could be correlated with the dose of radiation received.

4. Radiation of nuclear suspensions of thymus led to a decrease in activity.

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REFERENCES

Allfrey, V. G., Mirsky, A. E. & Stern, H. (1955). *Advanc. Enzymol.* **16**, 411.
Aronson, D. L., Fraser, M. J. & Smith, C. L. (1956). *Radiation Res.* **5**, 225.
Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Brown, G. L. (1952). *Biochem. J.* **51**, 569.
Burton, K. (1956). *Biochem. J.* **62**, 315.
Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951). *J. biol. Chem.* **191**, 447.
Creasey, W. A. (1960). *Biochim. biophys. Acta*, **38**, 181.
Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.
Dale, W. M. &. Russel, C. (1956). *Biochem. J.* **62**, 50.
DiCarlo, F. J. & Schultz, A. S. (1949). *J. biol. Chem.* **180**, 329.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Dounce, A. L. (1950*a*). In *The Enzymes*, vol. 1, part 1, p. 187. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Dounce, A. L. (1950*b*). *Ann. N.Y. Acad. Sci.* **50**, 982.
Dounce, A. L. (1952). *Exp. Cell Res.* **3**, Suppl. 2, 103.
Dounce, A. L. (1954). *Int. Rev. Cytol.* **3**, 199.
Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Dounce, A. L. & Monty, K. J. (1955). *J. biophys. biochem. Cytol.* **1**, 155.
Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.
Eichel, H. J. & Spirtes, M. A. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 412.
Euler, H. von & Hevesy, G. (1942). *Det. Kgl. Danske Videnskabernes selskab. Biol. Medd.* **17**, no. 8.
Feinstein, R. N., Butler, C. L. & Hendley, D. D. (1950). *Science*, **111**, 149.
Forssberg, A. (1947). *Nature, Lond.*, **159**, 308.
George, P. (1953). *J. biol. Chem.* **201**, 413.
Glock, G. E. & McLean, P. (1957). *Biochem. J.* **65**, 413.
Goutier-Pirotte, M. & Thonnard, A. (1956). *Biochim. biophys. Acta.*, **22**, 396.
Gray, D. E. & Deluca, H. A. (1956). *Amer. J. Physiol.* **184**, 301.
Heppel, L. A. (1955). In *Methods in Enzymology*, vol. 2, p. 570. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Heppel, L. A. & Hilmoe, R. J. (1955). In *Methods in Enzymology*, vol. 2, p. 546. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hilmoe, R. J. & Heppel, L. A. (1957). *J. Amer. chem. Soc.* **79**, 4810.
Hogeboom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Hotchkiss, R. D. (1948). *J. biol. Chem.* **175**, 315.
Huggins, C. &. Talalay, P. (1945). *J. biol. Chem.* **159**, 399.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 429.
Kaplan, J. G. & Paik, W. K. (1957). *J. gen. Physiol.* **40**, 147.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Kornberg, A., Lieberman, I. & Simms, E. S. (1955). *J. biol. Chem.* **215**, 389.
Kornberg, A. & Pricer, W. E. (1950). *J. biol. Chem.* **182**, 763.
LePage, G. A. & Umbreit, W. W. (1943). *J. biol. Chem.* **148**, 255.
Methods of Vitamin Assay (1951), p. 81. Ed. by the Assoc. of Vitamin Chemists. New York: Interscience Publishers Inc.
Miller, N. (1950). *J. chem. Phys.* **18**, 79.
Mori, K., Momoki, S. & Itô, H. (1951). *Igaku to Seibutsugaku*, **18**, 303. Quoted in *Chem. Abstr.* **45**, 9580.
Morton, R. K. (1958). *Nature, Lond.*, **181**, 540.
Needham, D. M. (1942). *Biochem. J.* **36**, 113.
Payne, A. H., Kelly, L. S. & Entenman, C. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 698.
Potter, R. L. & Grannis, G. F. (1953). *Fed. Proc.* **12**, 399.

Preiss, J. & Handler, P. (1957). *J. Amer. chem. Soc.* **79**, 4246.
Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Roth, J. S. & Milstein, S. W. (1952). *J. biol. Chem.* **196**, 489.
Siebert, G. & Smellie, R. M. S. (1957). *Int. Rev. Cytol.* **6**, 383.
Stern, H. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 177.
Stern, H. & Timonen, S. (1954). *J. gen. Physiol.* **38**, 41.
Sutton, H. C. (1956). *Biochem. J.* **64**, 447, 456.
Szent-Györgyi, A. (1926). *Biochem. Z.* **173**, 275.
van Bekkum, D. W. (1955). *Biochim. biophys. Acta*, **16**, 437.
Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1516.
Zatman, L. J., Kaplan, N. O. & Colowick, S. P. (1953). *J. biol. Chem.* **200**, 197.

Biochem. J. (1960) **77**, 12

Immunochemically-Active Cross-Linked Polystyrene Preparations

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In recent years the immune reaction has been used, directly or indirectly, for the fractionation of antibody proteins. Application of such methods has been reviewed by Isliker (1957). For example, Campbell, Luescher & Lerman (1951) have chemically conjugated antigen protein to a polymer such as cellulose and have used the product in a column to fractionate antibody. In the course of a more general attempt (Tozer, Cammack & Smith, 1958) to separate antigens by means of their immunochemical specificities, G. H. Whitham, B. T. Tozer & H. Smith (unpublished work) attempted to invert the method used by Campbell *et al.* (1951), and, by conjugating antibody to cellulose, use the product to fractionate antigens. The amount of antibody protein which can be conjugated in this manner is not large and the stoicheiometry of the immune reaction is such that the ratio of antigen combining with antibody is usually low. The result is that the capacity of such conjugated material is also low. It occurred to us that greater capacity could be achieved by using antibody protein to cross-link linear polymers such as a styrene, suitably substituted with a group capable of reacting with reactive centres or groups in the protein molecule e.g. tyrosyl and sulphydryl groups. While this work was in progress Gyenes, Rose & Sehon (1958) reported a similar approach to the fractionation of antibody proteins. The present paper describes the preparation of two types of such cross-linked polymers from styrene and antibody protein, in which the chemical conjugation is achieved in one by means of a diazo group and in the other by means of a mercury atom. A preliminary account of some of this work has already been published (Kent & Slade, 1959).

MATERIALS AND METHODS

Antigens. Pasteurella pestis toxin was prepared by $(NH_4)_2SO_4$ precipitation from the protein fraction of *Pasteurella pestis* grown on a casamino acid–glucose medium at 28° (Englesberg & Levy, 1954). Anthrax protective antigen was prepared as a culture filtrate prepared by the method of Thorne & Belton (1957). Ovalbumin was prepared by Na_2SO_4 precipitation according to the method of Kekwick & Cannan (1936).

Antibodies. Ovalbumin antiserum was prepared in rabbits and supplied by Dr H. Smith and Dr B. T. Tozer. Plague antiserum was prepared in rabbits by Dr A. P. MacLennan and was active against the toxin and several other, but not all, known antigens of *Pasteurella pestis*; 1 ml. of antiserum neutralized 4 mg. of toxin. A hyperimmune anthrax antiserum was prepared in horses by Mr F. C. Belton using a spore vaccine (Sterne Strain).

γ-Globulins. These were prepared from both normal and immune sera by Na_2SO_4 precipitation by the method of Kekwick & Record (1941) and stored as freeze-dried powders.

Polyaminostyrene. In the initial experiments linear polystyrene, Distrene 80 (British Resin Products Ltd. Hayes, Sully, Penarth, Glamorgan, Wales) was nitrated with a mixture of HNO_3 and H_2SO_4 to the extent of approximately one nitro group per aromatic ring; the nitro compound was reduced with $SnCl_2$ and HCl by the method of U.S. Pat. 2,366,008. The polyaminostyrene so prepared, though fully soluble in dil. HCl, gave, surprisingly, a sparingly soluble diazonium salt. This was traced to the presence in the product of a small percentage of sulphonic acid groups, derived from the H_2SO_4 used during the nitration. When HNO_3 alone was used in this stage, a soluble diazonium salt was formed. The method finally adopted was as follows.

Granular Distrene (20 g.) was mixed with 100 ml. of fuming HNO_3 (95%, w/w; sp.gr. 1·5) and warmed on a water bath until dissolved. Heating at 100° was then continued on the steam bath (usually for about 30 min.) until

a test sample, obtained by precipitation with water, washed well and dried, showed a nitrogen content of 9–10% by the Kjeldahl method modified for nitro compounds (Pregl, 1945). The product was then precipitated with water, washed well, drained and transferred to a flask fitted with a reflux condenser; $SnCl_2$ (200 g.) and conc. HCl (300 ml.) were added and the mixture was slowly heated to reflux on the hot-plate for 4 hr. We are indebted to Dr P. Jackson for informing us that this reaction may become violent, even in the later stages after the initial vigorous reaction has subsided. Almost all the solid dissolved to give a light-brown solution of the amine hydrochloride. The solution was filtered through a large sintered-glass filter (porosity 2 or 3) and the small residue was washed with a little water.

A solution of NaOH (300 g./300 ml. of water) was added slowly to the cooled filtrate with stirring. The polyaminostyrene was precipitated as a dirty white solid, whereas the tin hydroxides at first precipitated redissolved in the excess of alkali. The polyaminostyrene was collected by filtration through a thick layer of cellulose pulp on a large sintered-glass funnel (porosity 2) and washed well with N-NaOH solution until it was largely free of tin salts. The precipitate was protected from strong light and from the air by keeping it covered with liquid as much as possible to prevent its darkening. It was drained, and the cake of polyaminostyrene separated from the filter-paper bed as completely as possible and redissolved in warm 2N-HCl containing 10% of NaCl. The solution was adjusted to pH 1·0–1·5 and the tin was precipitated as sulphide by a stream of H_2S and removed by filtration through a layer of filter-paper pulp in a sintered-glass funnel. The solution was freed from H_2S by heating gently on a hot-plate in a stream of N_2. The yield could be calculated by evaporating a known volume to dryness, drying at 105–110° to give total solids and subjecting the residue subsequently to ignition, which gave the NaCl content. If the NaCl ash contains any Sn, the precipitation of free polyaminostyrene with excess of NaOH should be repeated, followed by dissolving again in 2N-HCl. A final concentration of 2% of polyaminostyrene was convenient.

Polystyrylmercuric acetate. Mercuric oxide (25 g.) was dissolved in 22 ml. of 72% perchloric acid (sp.gr. 1·7) and the solution was cooled and diluted slowly, with cooling and stirring, with 200 ml. of amyl acetate. Distrene (10 g.) was dissolved in 400 ml. of amyl acetate by gently warming on a water bath and the solution of mercuric perchlorate was added dropwise, with vigorous stirring, until any sticky precipitate that formed had redissolved. The pale-yellow solution was allowed to stand at 20°; a cream-coloured gelatinous precipitate formed and the reaction appeared to reach completion in 2 hr. The precipitate was filtered off and washed with amyl acetate until a test portion remained clear on dilution with 90% ethanol; it was then washed with 90% ethanol until it was free of amyl acetate and wetted by successive washing with 50% ethanol, 20% ethanol and, finally, with water.

The resulting polystyrylmercuric perchlorate was converted into the acetate by stirring with successive portions of aqueous 25–30% sodium acetate solution, to which a few drops of acetic acid had been added, until the filtrate after treatment was free of perchlorate. The solid was washed free of sodium acetate, drained well on the filter pump and stored in the damp state.

The mercury content of the product varied somewhat from batch to batch but was normally between 50 and 60%, based on a dried sample.

Conjugation with antibody protein

By means of polyaminostyrene. In general it is advantageous to use a weight of polymer approximately equal to that of the protein used.

A 2% solution of polyaminostyrene in 2N-HCl (10 ml.) was cooled to between 0 and −5°, diazotized by dropwise addition of 1·5 ml. of 14% $NaNO_2$ solution and kept at about −5° for 20 min. The excess of nitrous acid was destroyed by adding 2 ml. of 6% urea solution (previously cooled to 0°) and keeping the mixture between 0 and −5°, with occasional stirring, for about 1 hr. until a test drop failed to turn a moistened starch–iodide paper blue. The solution was cooled in ice–NaCl to about −10°, and 12 ml. of N-NaOH (also previously cooled to −10°) was added slowly with stirring. The stirring must be thorough and the pH must not rise at this stage above 4 or cross-coupling, with resulting precipitation, will occur. The protein solution (0·20 g. in 1% NaCl solution) and crystalline sodium acetate (1 g.) were quickly added, with stirring, when the pH rose to about 6 and a brown precipitate of polystyrylazoprotein began to form. The mixture was stirred gently overnight at 0°; the solution was adjusted to pH 8 with a few drops of dilute Na_2CO_3 solution and again stirred gently for 24 hr. at 0°. Normally all the protein will have been removed from the supernatant at this stage, as shown by a negative phosphotungstic acid test. The precipitate was then filtered off through a sintered-glass filter (porosity 3 or 4), well washed with 0·9% NaCl, resuspended in 0·9% NaCl and stirred successively at room temperature with further small amounts (5–10 mg.) of protein until the latter was no longer absorbed from solution. Any unchanged diazonium groups were then inactivated by stirring the solution with 0·2 g. of resorcinol in 5% sodium acetate solution for 24 hr.; the filtered product was washed with 0·9% NaCl until the filtrate was free from resorcinol. Preparations required to show the highest degree of specificity of adsorption were then allowed to stand for a further 24 hr. in 0·9% NaCl containing a few milligrams of the protein used and finally washed with 0·9% NaCl and stored in the same solvent.

For brevity, such preparations are referred to below as diazo 'resins', qualified by the word 'immune' if they contain antibody protein.

By means of polystyrylmercuric acetate. Damp polystyrylmercuric acetate, equivalent to about 10 times (dry weight) as much polymer as protein to be treated, was transferred to a metal or plastic container and shaken vigorously for ½ hr. with a glass bead and sufficient 5% sodium acetate solution to give a thin creamy suspension. The γ-globulin or other protein solution, containing 10–40 mg. of protein/ml. in 5% sodium acetate solution, was added and the mixture was stirred gently at 0–5° for 48 hr. If all the protein had reacted, as shown by a negative phosphotungstic acid test on the supernatant, the mixture was stirred with successive small quantities (5–10 mg.) of protein until some remained in the supernatant. The precipitate was filtered off. Any unchanged mercury groups were inactivated by stirring for 24 hr. at 0–5° with a M-solution of monothioethylene glycol in 5% sodium acetate, 2 ml./g. (dry wt.) of 'resin' being used. At the end of this

treatment the pH should be between 5·5 and 6·5 and the supernatant should contain excess of thiol, as shown by its ability to decolorize 0·01 N-iodine solution; but the 'resin' itself, after thorough washing, should fail to give a blue colour when a sample is treated on a white tile with a drop of diphenylcarbazone reagent (0·1 % in ethanol). The 'resin' was then washed with 0·9 % NaCl, stirred again for 24 hr. with 10 mg. of protein in 0·9 % NaCl, the mixture was filtered, the residue was washed with 0·9 % NaCl and stored at 0–5° in 0·9 % NaCl.

Such preparations are referred to below as mercury 'resins', qualified by the word 'immune' if they contain antibody protein.

Analytical methods

Total nitrogen. This was determined by the Kjeldahl method.

Protein. This was determined from the total nitrogen, estimation by a modified Sakaguchi–Weber (Weber, 1930) technique (Scanes & Tozer, 1956), or quantitative ninhydrin (Cocking & Yemm, 1954) on 6 N-HCl hydrolysates (100°; 18 hr.) by using the appropriate protein as standard. The last-mentioned method was usually used by way of confirmation in view of the possibility of producing traces of NH_3, but the results obtained always agreed well with those from the other two methods.

Mercury. This was determined by Kjeldahl decomposition and colorimetric estimation by (1) diphenylcarbazone (Snell & Snell, 1949) and (2) H_2S in the presence of gum (Snell & Snell, 1949).

Quantitative precipitin test. Antigen was determined by total N determinations on the specific precipitate according to the method of Heidelberger, Sia & Kendall (1930).

Antigen assays. Anti-anthrax protective antigen was assayed both by a neutralization technique against a standard antiserum on agar-diffusion plates by the method of Thorne & Belton (1957) and by a rabbit-skin test devised by Belton & Henderson (1956).

Pasteurella pestis toxin was determined by assay for toxicity in white mice, the LD_{50} being between 15 and 20 μg.

RESULTS AND DISCUSSION

Composition of the 'resins'

The polyaminostyrenes from which the diazo 'resins' were prepared had nitrogen contents between 10·0 and 11·2 % ($[\text{-CH}(C_6H_4\cdot NH_2)\cdot CH_2\text{-}]_n$ requires N, 11·7 %). The polydiazonium salts derived from the nitration with a mixture of nitric acid and sulphuric acid were capable of combining with 20–30 % by weight of protein. When nitric acid alone was used the capacity for protein was increased to 60–70 % by weight of the final 'resin'. The product, though softer than the normal ion-exchange resin is nevertheless easily handled. When drained on a sintered-glass funnel, the moist 'resins' contain as much as 80–90 % by weight of water. In order to minimize the risk of protein denaturation, these 'resins' were always stored and handled in the moist state, usually suspended in dilute electrolyte.

The introduction of mercury, by the method given, readily yielded products containing 55 % of mercury ($[\text{-CH}(C_6H_4\cdot HgOAc)\cdot CH_2\text{-}]_n$ requires Hg, 55·1 %). The solubility characteristics of the perchlorate formed initially suggest that some degree of cross-linkage occurs during the process. This is probably why the capacity of the modified polystyrene for protein is considerably lower than that of polydiazostyrene. The protein content varied between 12 and 20 %, calculated on the dry weight of 'resin', with a mean of about 15 %. These 'resins' also held considerable amounts of moisture. The moist product, after draining on a sintered-glass filter, usually contained 50–70 % by weight of water. The 'resins' were stored as moist powders.

Adsorption characteristics of 'resins'

From the physical constitution of these 'resins' some degree of non-specific adsorption can be expected, owing partly to physical factors and to non-specific protein–protein interaction. In an attempt to assess the capacity of these 'resins' for specific adsorption, tests were made with proportions of antibody and antigen in the region of the equivalence zone, determined from the precipitin curve (ovalbumin), neutralization tests (*P. pestis* toxin) or by direct absorption tests with diffusion plates (anthrax antigen). Except where a pure antigen is used, it is difficult to assign numerical values to the specific-adsorption efficiency of these resins, owing to the inherent inaccuracies of both bioassay and immunochemical assay of specific protein. Nevertheless it is not difficult to demonstrate a semi-quantitative selectivity of an immune 'resin' for its homologous antigen. It is much more difficult to demonstrate complete specificity (i.e. that the 'resin' takes up the appropriate antigen and that alone, from a mixture of proteins). To obtain a measure of non-specific adsorption we compared the behaviour of the immune 'resins' with that of exactly similar 'resins' prepared with γ-globulin from a normal animal.

To test these 'resins' we chose three systems of increasing complexity, using as antigen (*a*) ovalbumin (a pure antigen) in buffer solution, (*b*) the protective antigen against anthrax in a culture filtrate (small quantities of a well-characterized antigen in the presence of much other protein) and (*c*) a protein fraction from *P. pestis*, containing a toxin active against mice (LD_{50} 15–20 μg.) but also significant though minor amounts of other antigens.

Specificity. A sample of a diazo 'resin', made from anti-ovalbumin serum and containing 120 mg. of γ-globulin (1·5 times the theoretical quantity), when treated with 2 mg. of ovalbumin in 2 % potassium bicarbonate solution, removed 82 % of protein as shown by the quantitative precipitin

test. With double the quantity of 'resin' only traces of protein were left in solution. Under comparable conditions, the same 'resin' removed only 9·2 and 10·0 % of bovine-serum albumin from solution, in duplicate experiments. In another experiment a mercury 'resin', containing 48 mg. of anti-ovalbumin γ-globulin, when treated with a solution containing 0·75 mg. of ovalbumin, removed 93 % of the latter. Double amounts of 'resin' removed all but traces of ovalbumin. This 'resin' also removed only 10 % of serum albumin under comparable conditions.

The protective antigen against anthrax behaves abnormally in the precipitin test, and from neutralization data it is difficult to assess the zone of equivalence. In consequence, the equivalent proportions of antibody to antigen were determined by successive additions of immune 'resin' to a standard volume of culture filtrate until no antigen was detectable in the supernatant by the agar-diffusion-plate technique. An amount of immune diazo 'resin' containing 3·5 mg. of γ-globulin was required to neutralize 1 ml. of culture filtrate, whereas with an immune mercury 'resin' an amount containing 1·5 mg. of γ-globulin was equivalent to 1 ml. of filtrate. This difference suggests that the diazo type of coupling reduces the capacity of antibody for antigen. In approximately these proportions both the diazo and mercury immune 'resins' remove the protective antigen completely from culture filtrate without significant decrease in the total protein content (quantitative Sakaguchi estimation).

Amounts of immune 'resin' containing 8 mg. of γ-globulin would remove 1 mg. of *P. pestis* toxin from solution, a figure in approximate agreement with the neutralization data. But somewhat larger quantities of cellulose pulp, kieselguhr or even powdered azobenzene would do the same. Our earlier work with this toxin was largely vitiated by its adsorption on sintered-glass filters. Even millipore filters have a small but significant capacity for adsorbing the toxin. Evidently non-specific adsorption is of great importance and our efforts were concentrated on decreasing it to the minimum (see below).

In order to assess the magnitude of the effect of non-specific adsorption, the adsorptive capacity of 'resins' containing γ-globulin from normal animals has been compared with that of the immune 'resins' under identical conditions. After adsorption, the 'resin' is washed well with buffer (1 % sodium chloride–1 % phosphate buffer, pH 7·8) and 5 % potassium bicarbonate solution and the total filtrate assayed.

An amount of diazo 'resin', containing 400 mg. of normal γ-globulin (twice the equivalent amount of immune 'resin') removed only 10 % of ovalbumin from a 0·9 % sodium chloride solution containing 3 mg. of ovalbumin and only a trace from a similar solution in 5 % potassium bicarbonate. In a similar experiment an amount of mercury 'resin' containing 120 mg. of normal bovine-serum γ-globulin removed no significant amounts of ovalbumin from a solution containing 2·5 mg. in potassium bicarbonate solution (total nitrogen and quantitative precipitin test), whereas a mercury 'resin' containing the same quantity of anti-ovalbumin γ-globulin removed 85 % (quantitative precipitin), or 80 % (total nitrogen) from a similar solution.

Similarly, amounts of both 'resins' containing 10 mg. of normal, horse γ-globulin (three times the equivalent for an immune diazo 'resin', six times the equivalent for an immune mercury 'resin') removed no detectable amount of anthrax protective antigen from 1 ml. of culture filtrate, as judged by diffusion-plate assays. Although this assay method might not detect even as much as a 20 % decrease in antigen, the use of considerably greater amounts of normal 'resin' than of immune 'resin' emphasizes that non-specific adsorption is small.

The position was markedly different with *P. pestis* toxin. This was chosen as an antigenically complex material, about which much was known. The antiserum used was active against other components in addition to toxin but reacted weakly with antigens 3 and 4 of Crumpton & Davies (1956). The experiments with immune 'resins' reported above showed also that much protein was adsorbed with the toxin. The amounts were such as to suggest strongly that non-antigenic components were being removed. This was confirmed by experiments with 'resins' containing normal γ-globulin. With amounts of both types of 'resin' equal to the amounts of immune 'resin' required to remove all antigen from solution, non-specific adsorption to the extent of 20–30 % of the toxin occurred regularly, accompanied by at least an equivalent proportion of total protein. Potassium bicarbonate (5 % solution) appeared to be the best suspending and washing medium, and with this solution a non-specific adsorption as low as 15 % was recorded in one experiment.

Attempts have been made to decrease the magnitude of the non-specific adsorption. It is not due solely to protein–protein interactions, since 'resins' containing only 20 % of γ-globulin show a similar effect to those containing 60 %. Methods other than the action of resorcinol were tried for removing unchanged diazonium and mercury groups. These included the action of diethylamine and of cuprous chloride for diazo groups, and methanethiol and thioglycollic acid for the mercury 'resins', but the non-specific adsorption was not

decreased. It seems therefore that this ready adsorbability is a property of the antigenic preparation itself.

Biological activity of adsorbed antigen

Antigen adsorbed on immune 'resins' is firmly held. No systematic attempts have been made to release it, but it is not removed by prolonged washing with 0·9% sodium chloride or dilute buffers, including 5% potassium bicarbonate. It seemed important to attempt to determine whether the antigen had retained its biological activity after adsorption in this fashion. In view of the many data on the immunization levels for anthrax in experimental animals available in this Establishment, the anthrax protective antigen was chosen as the test case. Rabbits and guinea pigs were injected subcutaneously with suspensions of both types of immune 'resins' on which the protective antigen had been adsorbed. In all experiments an amount of antigen equal to that contained in 1 ml. of the culture filtrate (Thorne & Belton, 1957) was administered and the degrees of immunity produced were compared with those produced by the alum preparation of Belton & Strange (1954). This preparation, at 1 ml. of culture filtrate level, gives a high degree of protection in rabbits and a moderate degree of protection in guinea pigs if given in two doses 10 days apart, but a low degree of protection if given in one dose. The same total dose of antigen (half adsorbed on 'resin' and half on alum) was also administered in one dose. The animals were challenged with 200 times the median lethal dose (LD_{50}) of anthrax spores 21 days after inoculation. The results are shown in Table 1.

Table 1. *Comparison of 'resin' preparations containing anthrax antigen with an alum preparation (Belton & Strange,* 1954)

The numbers of animals surviving injection of the preparations shown in the first column and subsequent challenge with 200 lethal doses of anthrax spores are given in columns 3 and 4.

Form of antigen	No. of doses	Animals surviving challenge: Rabbits	Animals surviving challenge: Guinea pigs
Alum	2	4/4	6/10
Alum	1	1/4	0/10
Diazo 'resin'	1	3/4	4/10
Hg 'resin'	1	3/4	1/4*
Diazo 'resin' + alum	1	3/4	5/10
Hg 'resin' + alum	1	4/4	6/10
Controls			
0·9% NaCl	1	0/4	0/10
Diazo 'resin' without antigen	1	0/4	0/10
Hg 'resin' without antigen	1	0/4	0/4*

* Six guinea pigs died before challenge.

The immune 'resins' without antigen were included as controls to eliminate the remote possibility that the small amount of bound antibody administered was establishing a degree of passive immunity. The mercury preparations proved somewhat toxic for guinea pigs, killing six out of ten when administered with antigen or without it as a control (Table 1). Half the quantity of mercury 'resin' administered with alum produced no deaths. Dr M. C. Lancaster kindly carried out autopsies on the guinea pigs that died before challenge; their condition was typical of heavy-metal intoxication. None of the rabbits showed any such symptoms. All deaths after challenge were confirmed as due to anthrax infection.

A few experiments have also been carried out to determine the antibody response in sheep to anthrax antigen adsorbed on these 'resins'. An amount of antigen equal to that in 5 ml. of culture filtrate (Thorne & Belton, 1957) was administered intramuscularly: (*a*) adsorbed on an immune mercury 'resin'; (*b*) adsorbed on an immune diazo 'resin'; (*c*) as an alum precipitate both in one and in two doses. The antibody levels in the sera after 3 and 6 weeks were determined by their abilities to neutralize a standard amount of anthrax toxin in the rabbit-skin test of Belton & Henderson (1956) and of standard amounts of antigen in the agar-diffusion-plate test of Thorne & Belton (1957). As expected, single doses of the alum preparation produced no antibody response detectable by either method of assay, whereas in two doses satisfactory levels were attained. The mercury 'resin' with adsorbed antigen in a single dose gave levels comparable with the alum preparation in two doses. The diazo 'resin' in one dose gave a positive response in only one animal out of six. This suggests not only that diazo coupling decreases the capacity of the antibody for antigen but also that the latter may be less tenaciously held. We have also found (unpublished observation) that diazo coupling tends to decrease the combining power of antibody for its homologous antigen.

SUMMARY

1. Highly swollen linear chains of polystyrene have been cross-linked by means of antibody protein, by using diazo coupling and coupling via the sulphydryl group of the protein by means of a mercury atom.

2. Preparations containing 15% to over 60% of protein, by weight, are described.

3. Such products are able selectively to adsorb their appropriate antigen from solution.

4. With two antigens out of the three investigated, namely ovalbumin and anthrax protective antigen, the action is markedly specific, only small

quantities of heterologous protein being adsorbed. With the third antigen, *Pasteurella pestis* toxin, non-specific adsorption is substantial.

5. With the anthrax preparation, the antigen, when adsorbed on the polymer, retains its biological competence as judged by its ability to stimulate antibody production and to produce immunity to challenge in animals after injection.

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REFERENCES

Belton, F. C. & Henderson, D. W. (1956). *Brit. J. exp. Path.* **37**, 156.
Belton, F. C. & Strange, R. E. (1954). *Brit. J. exp. Path.* **35**, 144.
Campbell, D. H., Luescher, E. & Lerman, L. S. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 575.
Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
Crumpton, M. J. & Davies, D. A. L. (1956). *Proc. Roy. Soc.* B, **145**, 109.
Englesberg, E. & Levy, J. B. (1954). *J. Bact.* **67**, 438.
Gyenes, L., Rose, B. & Sehon, A. H. (1958). *Nature, Lond.*, **181**, 1465.
Heidelberger, M., Sia, R. H. P. & Kendall, F. E. (1930). *J. exp. Med.* **52**, 477.
Isliker, H. C. (1957). *Advanc. Protein Chem.* **12**, 387.
Kekwick, R. A. & Cannan, R. K. (1936). *Biochem. J.* **30**, 227.
Kekwick, R. A. & Record, B. R. (1941). *Brit. J. exp. Path.* **22**, 29.
Kent, L. H. & Slade, J. H. R. (1959). *Nature, Lond.*, **183**, 325.
Pregl, F. (1945). *Quantitative Organic Microanalysis*, 4th English ed., p. 82. London: J. and A. Churchill Ltd.
Scanes, F. S. & Tozer, B. T. (1956). *Biochem. J.* **63**, 565.
Snell, F. D. & Snell, C. T. (1949). *Colorimetric Methods of Analysis*, 3rd ed., vol. 2, pp. 73, 75. New York: van Nostrand Co. Inc.
Thorne, C. B. & Belton, F. C. (1957). *J. gen. Microbiol.* **17**, 505.
Tozer, B. T., Cammack, K. A. & Smith, H. (1958). *Nature, Lond.*, **182**, 668.
Weber, C. J. (1930). *J. biol. Chem.* **86**, 217.

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The Chemical Nature of the Toxic Compounds Containing Fluorine in the Seeds of *Dichapetalum toxicarium*

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A fluoro-octadecenoic acid has been isolated in small amounts from the seeds of *Dichapetalum toxicarium* (Peters & Hall, 1959). It was obtained by fractionation of the oil soluble in acetone at $-20°$ on silane-treated Hyflo Supercel columns with liquid paraffin and aqueous acetone as the stationary and mobile phases (Howard & Martin, 1950; Crombie, Comber & Boatman, 1955). In this Laboratory, the fraction from the columns eluted by aqueous 55% (v/v) acetone was purified by precipitation as the calcium salt. However, analysis showed that, with one exception, all our small samples were contaminated with 5–10% of an impurity. Reversed-phase paper chromatography showed that this fraction contained up to three components and it was clear that the structure of the fluoro-octadecenoic acid could not be determined finally by this method. The present paper describes an alternative method of preparing gram quantities of the pure fluoro fatty acid, and the determination of its structure as fluoro-oleic acid (ω-fluoro-*cis*-Δ^9-octadecenoic acid). During the final stages of the purification another fluorine-containing component was found in small amounts. Unlike fluoro-oleic acid this is a solid at room temperature and its chemical structure has not yet been determined.

MATERIALS AND METHODS

Special chemicals. The activated charcoal was from Hopkin and Williams Ltd. and was previously washed with light petroleum (b.p. 40–60°). Linolenic acid, linoleic acid and oleic acid were from Nutritional Chemicals Ltd. and

elaidic acid was from K and K Laboratories Ltd. Embacel was from May and Baker Ltd. and Reoplex 400 from The Geigy Co. Ltd. Oil Red 4B was from George T. Gurr Ltd. The cellulose powder was Whatman Ashless Standard Grade. Samples of ω-fluoro-octanoic acid, ω-fluoro-nonanoic acid and ω-fluorodecanoic acid were kindly presented by Dr F. L. M. Pattison. Other chemicals were of A.R. quality where possible.

Analytical methods

Double-bond estimations. These were made essentially by Trappe's (1938) method, except that the bromination was carried out in bromine–methanol without $CHCl_3$, and N-HCl (1 ml.) together with the 2% (w/v) KI (1 ml.) was added after the bromination was complete.

Carboxyl titration. A blank titration, consisting of 2 ml. of 90% (v/v) ethanol containing 20 μl. of 0·04% phenol red, was titrated with 5 mM-NaOH in aqueous 90% (v/v) ethanol until a bright-red colour persisted for at least 2 min. During this time a fine stream of CO_2-free air was passed through the solution. Up to 0·5 mg. of the fluoro acid in 0·2 ml. or less of carbon tetrachloride was then added to the blank, and the titration continued as before. The end point was sensitive to 2–3 μl. of 5 mN-NaOH in a titration of 300–400 μl. Micropipettes and small-volume Ostwald–van Slyke pipettes were used for the volume measurement of the samples. The titration was made with a Conway horizontal microburette with the tip below the surface of the solution.

Fluorine estimation. The fluorine from a long-chain ω-fluoro fatty acid in our experience cannot be split off by heating with 7·5N-KOH, though we can confirm that 7·5N-NaOH or -KOH will remove the fluorine from fluoroacetate (Saunders, 1957*a*; Mirosevic-Sorgo & Saunders, 1959). Some digestion in a Parr-type bomb is therefore necessary. Many attempts were made to obtain reproducible values for NaF when added to such a bomb and heated at 500°. Though new bombs gave reasonable recoveries at first, after a few analyses poor results were obtained. To overcome these difficulties the surface of the nickel bomb chamber was kept polished, 14-carat-gold washers were used instead of 9-carat and the surfaces were polished after each digestion, sodium peroxide was used instead of metallic sodium, and calcium nitrate was added to fix the fluorine during the digestion and to oxidize the residual carbon.

Samples were prepared for fluoride titration as follows. The purified oil (3–5 mg.), in carbon tetrachloride, was transferred to the nickel bomb chamber and the solvent evaporated away with a gentle stream of compressed air. To the oil was added 1 ml. of 10% (w/v) $Ca(NO_3)_2$ in acetone. The acetone was removed with compressed air. Sodium peroxide (100 mg.) was added and the bomb sealed and placed in a muffle furnace at 500–550° for 2 hr., and cooled in the muffle. The residue in the bomb, a white solid, was first extracted with 3–4 ml. of N-perchloric acid. The bomb was then washed out with water, the volume of the digest solution was made to nearly 250 ml. and the pH of the contents adjusted to approx. pH 6·0 with N-NaOH. The volume was made up to 250 ml. and any insoluble material was removed by centrifuging. Fluoride was determined by thorium-titration as described by Hall (1957) except that: 1 mN-thorium nitrate was used, the buffered alizarin sulphonate reagent was adjusted with 7–8 ml. of 1 mN-thorium nitrate for every 100 ml. of alizarin reagent (this step obviated the need for a blank thorium titration), and the titration was scaled down so that not more than 8 μg. of fluorine was titrated, with a reproducibility of ±0·01 ml. of 1 mN-thorium nitrate.

Qualitative analysis by reversed-phase paper chromatography. This was based upon the method of Schlenk, Gellerman, Tillotson & Mangold (1957) for the separation of C_{16}–C_{22} fatty acids. Instead of sheets of Whatman no. 1 filter paper treated with silicone, the fast hard paper Whatman no. 531 was used and liquid paraffin substituted for silicone. Sheets 46 cm. × 19 cm. were soaked in a 7% (v/v) solution of liquid paraffin in ether and dried in air. Traces of impurities were then removed by washing with aqueous 85% (v/v) acetic acid by descending chromatography for 48 hr. The papers were then dried at about 50°. Spots of 5 μl. of the fractions for analysis were put on the paper 3 cm. from one end. The chromatograms were developed at room temperature by ascending 85% acetic acid for 12–18 hr. and dried for 15 min. at 50°. The papers were suspended in iodine vapour, when the unsaturated fatty acids rapidly appeared as dark-brown spots (Brante, 1949). The method will detect a few micrograms of an unsaturated fatty acid.

Inhibition of citrate metabolism induced in guinea-pig-kidney particles. Guinea-pig-kidney particles (Peters & Wakelin, 1957) were suspended in a mixture of 1% (w/v) KCl solution and 0·1M-KH_2PO_4 neutralized to pH 7·2 with NaOH. Citrate accumulation in the particles was induced by the fluoro acids as follows. A mixture of kidney-particle suspension (1·9 ml.), 0·8% $MgCl_2,6H_2O$ solution (0·1 ml.), adenosine triphosphate (0·33 mg. of disodium salt in 0·1 ml.), trisodium citrate (10 μmoles in 0·4 ml.), fluoro acid as the sodium salt in 0·2 ml. and 1% (w/v) KCl solution (0·3 ml.) was incubated for 30 min. at 38°. After incubation aqueous 25% (w/v) trichloroacetic acid solution (1 ml.) was added and solid material centrifuged off. Citrate was estimated in the supernatant solution by the method of Taylor (1953). A control experiment, with 1% (w/v) KCl solution in place of the fluoro acid solution, served as a standard against which the citrate inhibition induced by the fluoro acids could be measured.

Melting points. A B.S.S. thermometer corrected for the emergent stem was used. The heating bath contained about 700 ml. of ice-cold water, the temperature of which was raised at a rate of 0·5°/min. on a hot plate containing a magnetic stirrer.

Hydrogenation. A small sample (10 mg.) of the fatty acid was dissolved in 1·5 ml. of ethanol. To this were added 1·5 ml. of water and 2 ml. of a Raney nickel suspension in ethanol (Covert & Adkins, 1932). The reaction mixture was kept at 65–85° for 4 hr. with a slow stream of hydrogen bubbling through it. The Raney nickel was separated, and the hydrogenated fatty acid solution was acidified with 0·5 ml. of 2N-HCl and extracted with light petroleum (b.p. 40–60°).

EXPERIMENTAL AND RESULTS

The light-petroleum extract of the seeds of *D. toxicarium* was evaporated and saponified with methanolic KOH and partially purified with acetone at −20° (Peters & Hall, 1959). The resulting

oil, when analysed by reversed-phase paper chromatography, showed four major components. Two were oleic acid and linoleic acid (R_F 0·27 and 0·50 respectively), one the fluoro fatty acids (R_F 0·76) and the other a component running near the solvent front. The component containing the fluoro fatty acids was thought to be only fluoro-oleic acid, but in the final stages of the purification it proved to contain a small proportion of another fluoro acid which was a solid at room temperature. The term 'fluoro fatty acids' has been used to describe this component, to distinguish it from the purified fluoro-oleic acid and the solid fluoro acid.

Preparation of fluoro fatty acids by reversed-phase chromatography

Attempts to utilize filter-paper sheets for the quantitative preparation of the fluoro fatty acids were unsuccessful. Large amounts of the seed oil soluble in acetone at $-20°$ when put on the paper did not separate even when thick paper was employed. Complete separation was achieved by the use of cellulose-powder columns treated with liquid paraffin.

Location of the fluoro fatty acids. The principle was to follow the acids with some coloured substance which would travel at approximately the same rate. Several oil-soluble dyes were tested, and the only one suitable was Oil Red 4B (xylene–azotoluene–azo-β-naphthol). An orange component found in three samples of this dye moved ahead of the main dye and travelled with the fluoro acids. The initial separations were made with small cylindrical columns, which are also necessary when there is little of the material. Later the same principle was used on a much larger scale in an apparatus constructed of glass and Tufnol plates (Hall, 1960). By this method approx. 2 g. of the pure fluoro-oleic acid has been prepared from about 150 g. of crude fat obtained from 1 kg. of seeds.

Preparation of the cylindrical column. Cellulose powder (500 g.) was suspended in 2 l. of aqueous 85% (v/v) acetic acid. About 200 ml. of the mixture at a time was filtered under reduced pressure in a 4 in. sintered-glass funnel. The pad of powder was washed through once with approx. 200 ml. of acetic acid. The cellulose was resuspended in 2 l. of a 7% (v/v) solution of liquid paraffin in ether, and again filtered through the sintered funnel under reduced pressure. The cellulose was resuspended in 2 l. of 85% acetic acid to remove excess of paraffin and filtered again. Traces of acetic acid left in the cellulose were removed either by spreading the powder on a dish and drying in air or in an oven at 50°.

A tube of Pyrex glass 45 cm. × 10–12 mm. was used with a 100–120 μ sintered-glass disk 5 cm. from one end. Only sufficient of the paraffin-treated cellulose powder was introduced at a time to give a section approx. 1 cm. thick when compressed tightly with a glass rod which just fitted inside the column. To avoid irregular packing it was important that this rod should have an absolutely flat end at right angles to its length. The column was packed to within 5 cm. of its open end. At first the fatty acid mixture was applied in about 1 ml. of acetone, but the separation was unreliable. About 200 mg. of the crude fatty acids was evenly stained with 0·5 ml. of 0·1% (w/v) dye in ether and 1·5 g. of treated cellulose powder was added to this solution. The mixture was well stirred, transferred to the column and compressed with the glass rod so that a disk 3–5 mm. thick was formed. The packing of the column was completed to within 2 mm. of the open end and the packing finally covered with a circle of Whatman no. 531 filter paper. The column was clamped with the open end dipping into a beaker of solvent. Originally this was acetic acid–water (17:3, v/v), but later formic acid–acetic acid–water (2:2:1, by vol.) was used. With 85% acetic acid all the components separate but not widely; with formic acid present the travel of oleic acid, linoleic acid and linolenic acid is much retarded and the degree of contamination of the fluoro fatty acids is decreased. The column was developed by ascending solvent for 18 hr. at 18–25°.

The fluoro fatty acids moved with the fast-running orange component of the marker dye. The parts of the column that were not wanted were removed to within 5 cm. of the beginning of the orange section. A layer of approx. 1 cm. of new cellulose loosely packed was inserted and the column eluted with the developing solvent. Small volumes of eluate (0·2 ml.) were collected when the orange band started to come out, and these were continued until 2 ml. in all had been collected after the last of the dye had been eluted. The solvent was removed from each volume with a stream of air at room temperature or at about 35°. Each residue was examined by dissolving it in 0·5 ml. of carbon tetrachloride, and chromatographing 5 ml. portions on filter-paper sheets as described. Some of the earlier and later fractions contained small amounts of iodine-staining components other than the fluoro fatty acids. These were discarded. The fractions shown by paper chromatography to contain the fluoro fatty acids and no other components staining with iodine were pooled and the carbon tetrachloride was removed by evaporation.

Purification of the fluoro fatty acids

These fractions were contaminated with dye, liquid paraffin and non-acidic components of the seed oil of similar chromatographic mobility. To free the fluoro fatty acids from these contaminants they were precipitated as their calcium salts. The residues, in about 200 mg. quantities, after evaporation of the carbon tetrachloride were dissolved in 2 ml. of acetone and titrated with 0·25 N-NaOH to pH 8·0, any precipitate being removed by centrifuging. An equal volume of 10% (w/v) $CaCl_2$ (anhydrous) in aqueous 50% (v/v) acetone, previously adjusted to pH 8·0, was added, and the contents of the tube were centrifuged at 4000 rev./min. The precipitate was suspended in 2 ml. of 1% (w/v) $CaCl_2$ in 50% (v/v) acetone at pH 8·0 and centrifuged. The precipitate was washed twice with 5 ml. of light petroleum (b.p. 40–60°), which removed most of the orange dye and the washings were discarded. After a further centrifuging, the precipitate was emulsified with 2 ml. of 2 N-HCl and extracted twice with 2 ml. of light petroleum (b.p. 80–100°). After evaporation of the combined extracts to 1 ml., they were treated with 50 mg. of activated charcoal. The charcoal was separated by centrifuging and

washed with 1 ml. of light petroleum (b.p. 80–100°). The washing combined with the supernatant light petroleum was washed once with 5 ml. of water, made to 10 ml. and left in the deep freeze overnight at −25°. Any precipitate at this stage was removed by centrifuging at −25°, the supernatant solution was separated and the light petroleum removed by evaporation. The clear oil obtained after evaporation of the light petroleum, when frozen and brought slowly to 20°, became an opaque gel. Upon centrifuging at 24 000 *g* for 10 min. at 15–20° the gel separated into a clear supernatant oil and a white solid, which was another fluoro acid. The solid was separated and washed three times with 0·2 ml. of liquid paraffin, in which the fluoro-oleic acid is soluble. After the final washing the precipitate was dissolved with 2 ml. of 90 % (v/v) acetone to remove residual paraffin. The acetone solution of the solid phase was kept at −25°, when a crystalline precipitate formed which was centrifuged off at −25°. The precipitate was collected and dissolved in 1 ml. of acetone. When converted into the sodium salt, it was insoluble in acetone but soluble in warm water. The sodium salt was treated with HCl and the free acid extracted with light petroleum (b.p. 80–100°). After evaporation of the solvent the white solid was dissolved in absolute methanol and centrifuged to remove the last traces of liquid paraffin. Evaporation of the methanol gave 14 mg. of a white crystalline solid.

In later preparations, after the washing with liquid paraffin, the solid was dissolved in methanol (2 ml.), when most of the paraffin separated. After removal of the methanol by evaporation, the solid was redissolved in methanol (1 ml.) to separate the remaining paraffin and crystallized from light petroleum (b.p. 40–60°) at −25°.

Properties of fluoro-oleic acid

Table 1 gives the equivalent weight, the fluorine content and the number of double bonds.

Melting point. Some specimens melted at 12·5°; others softened at 16° and were not completely melted until 21°; the presence of quite small amounts of the solid fluoro fatty acid has a large influence. Our purest specimen of fluoro-oleic acid softened at 12·4° and melted at 13·5°. Another crystalline specimen melted at 18–22°. In this connexion, we have found that oleic acid containing 5 % (w/v) of stearic acid melts at 30° instead of 13·5°, whereas with 1 % it melted at 12° and with 2 % at 16–20°.

Position of the double bond and fluorine atom. The position of the double bond was determined by ozonization and identification of the degradation products by gas chromatography. The purified oil (12 mg.) was dissolved in 2 ml. of chloroform–acetic acid (2:1, v/v) and ozone was passed through the solution at 18–25°; losses by evaporation were made good. [The silver treatment described by Cason & Tavs (1959) was unsuitable.] After 16 hr. the solvents were removed in a current of air. The mixture was esterified with methanol and thionyl chloride (Carlson & Wadström, 1958) and examined by gas chromatography with a modified Martin

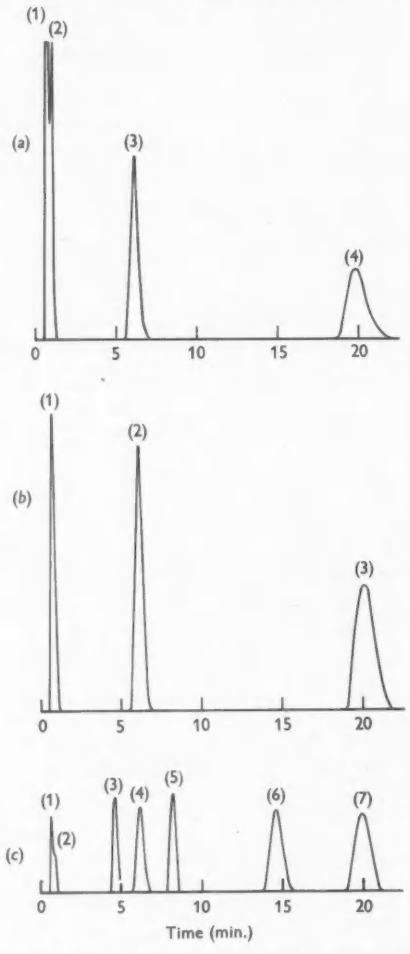

Fig. 1. Separation of methyl esters of fatty acids by gas chromatography at 197° on a 4 ft. 3 in. column with Reoplex 400 (40 %, w/w) as stationary phase and 60–80 mesh Embacel as support. N_2 pressure: 23 cm. Hg; N_2 flow: 120 ml./min. (*a*) Methyl esters of products of ozonolysis of fluoro-octadecenoic acid from *D. toxicarium*. Peaks in order of appearance: (1) ether, (2) methanol, (3) and (4) methyl esters of acids produced by ozonolysis. (*b*) Methyl esters of products of ozonolysis of fluoro-octadecenoic acid from *D. toxicarium* mixed with methyl ω-fluorononanoate and methyl azelate: (1) ether, (2) methyl ester of acid produced by ozonolysis + methyl ω-fluorononanoate, (3) methyl ester of acid produced by ozonolysis + methyl azelate. (*c*) Mixture of samples of methyl esters: (1) ether, (2) methanol, (3) methyl ω-fluoro-octanoate, (4) methyl ω-fluorononanoate, (5) methyl ω-fluorodecanoate, (6) methyl suberate, (7) methyl azelate.

Table 1. *Analysis of fluoro-oleic acid*

	Fluorine (%)	No. of double bonds	Equiv. wt.*
Specimen A	6·30±0·1	0·98±0·01	302±3
Specimen B	6·28±0·1	0·92±0·01	304±3
C_{18} fluoro-enoic acid requires	6·33	1·00	300

* Calculated from titration of CO_2H groups.

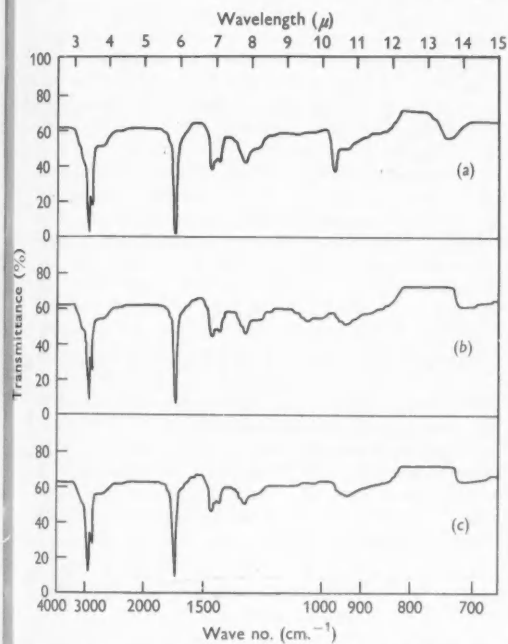

Fig. 2. Infrared spectra of (*a*) elaidic acid, (*b*) ω-fluoro-oleic acid and (*c*) oleic acid (5 %, w/v in carbon tetrachloride).

gas-density meter (Martin & James, 1956) constructed at Babraham. Figs. 1 (*a*) and (*c*) show that ozonization gave the methyl esters of azelaic acid and of ω-fluorononanoic acid. Hence the compound has a double bond between C_9 and C_{10}. The ω-position of the fluorine atom was proved by nuclear-magnetic-resonance determinations (see Appendix). With the evidence of the m.p. and the infrared spectrum the compound is ω-fluoro-oleic acid and not the *trans* form such as ω-fluoroelaidic acid. A further proof of the structure is given in Fig. 1 (*b*), where the products of ozonization were mixed with azelaic and ω-fluorononanoic acids. No additional chromatographic peaks separated.

Methyl ω-fluoro-oleate when chromatographed by the use of the Martin gas-density meter under the conditions used in Fig. 1 had a retention volume 2·7 times that for methyl oleate, showing that the fluorine atom has a large retarding influence.

Table 2. *Inhibition of citrate metabolism in guinea-pig-kidney particles induced by the fluoro acids*

The inhibition of citrate metabolism induced by the fluoro acids in the guinea-pig-kidney-particle system described in the text was measured after incubation for 30 min. at 38°. In all the values a control containing no fluoro acid has been subtracted. Control values were of the order of 0·3 μmole.

Additions	Wt. (μg.)	Citrate remaining (μmoles)
Sodium fluoroacetate	50·0	4·8
Fluoro-oleic acid	2·5	4·4
Unknown solid fluoro acid	2·5	4·3

Hydrogenation. The acid was hydrogenated with Raney nickel in ethanol at 65–85° and a product was formed with m.p. 68–71°. This was consistent with a conversion into fluorostearic acid, but the matter was not pursued as the results were not always reproducible.

Infrared and ultraviolet spectra. The infrared spectrum of fluoro-oleic acid was compared (Fig. 2) with the spectra of oleic acid and of elaidic acid with a Perkin–Elmer Infracord. The main difference between 5 % (w/v) solutions in carbon tetrachloride of oleic (*cis*) acid and elaidic (*trans*) acid was the peak for elaidic acid at 970 cm.$^{-1}$ (10·3 μ). This peak was absent from fluoro-oleic acid, which confirms that it is the *cis*-compound, in agreement with previous deductions by Mr L. C. Thomas (personal communication). The CF band shows up at 1050 cm.$^{-1}$. The ultraviolet spectrum of fluoro-oleic acid in ethanol was similar to that of oleic acid except for a small peak at 270–275 mμ.

Biological observations. A solution of fluoro-oleic acid in arachis oil (0·2 ml.) was injected intraperitoneally into rats. The lethal dose was 7–9 mg./kg. body wt. The amounts of fluoro-oleic acid producing citrate accumulation in guinea-pig-kidney particles were essentially the same as observed by Peters & Hall (1959). That is, 1·25 μg. of fluoro-oleic acid induced an accumulation of 2·6 μmoles of citrate and 0·62 μg. an accumulation of 1·2 μmoles. It was confirmed that the acid decreased citrate metabolism in the kidney particles much more than did fluoroacetate (Table 2).

Properties of the solid fluoro acid

This appeared to be not more than 5 % of the toxic oil obtained after purification by precipitation as the calcium salt. The acid had m.p. 72·0–73·7° (corr.) with softening at 70·5°.

It has no double bond and the carboxyl-group titration gave a value for the equivalent weight of 286. The fluorine content was 6·8 %. This could represent an acid with 17 carbon atoms but the acid inhibits aconitase in the guinea-pig-kidney-particle test (Table 2) and is toxic to rats; this indicates that it acts as an acid with an even number of carbon atoms (Peters, 1955) and is virtually as active as fluoro-oleic acid.

The infrared spectrum in carbon tetrachloride was similar to that of fluoro-oleic acid, except that the band at 1460 cm.$^{-1}$ was much more pronounced. The absence of the band at 970 cm.$^{-1}$ indicates that it is not a *trans*-isomer. The ultraviolet spectrum resembled that of oleic acid. The small peak at 270–275 mμ was absent.

DISCUSSION

The first experiments on the toxic fluoro oil from *D. toxicarium* (Peters, Wakelin, Birks, Martin & Webb, 1959) indicated that it was a long-chain fatty acid. The constitution of the main component is now proved, so far as this can be done without synthesis. That of the solid fluoro acid is not yet clear; its fluorine content, carboxyl-group titration and absence of CH=CH suggest a saturated acid with possibly 17 carbon atoms. The biochemical tests indicate that it behaves to the enzymes concerned as an acid with an even number of carbon atoms. This suggests either a branched-chain ω-fluoro acid with an even number of carbon atoms in the fluorine-containing chain or an ω-fluoro hydroxy acid with 16 carbon atoms. Hansen, Shorland & Cooke (1953, 1954) have shown that some branched-chain fatty acids found in mutton fat all have low optical rotations. With the small amount of the solid fluoro acid available, the optical rotation in propan-1-ol was not measurable, from which we concluded that the compound was optically inactive or had $[\alpha_D] < 2{\cdot}0$. This observation did not confirm the presence of a branched-chain structure or a hydroxyl group but the subject is being investigated.

The fluorine content of the soil in which plants that contain organic fluorine compounds grow raises some points requiring investigation. *Dichapetalum cymosum*, the leaves of which contain fluoroacetate, is stated to grow in a soil rich in fluorine (Saunders, 1957*b*). On the other hand, in a personal communication, Dr Dagmar Wilson said that she saw no evidence of fluorosis in Sierra Leone, where *Dichapetalum toxicarium* grows, from which she concluded that the soil content in this part was low in fluorine. If this is so, *Dichapetalum toxicarium* must concentrate fluorine compounds.

We now know the main compound responsible for the condition of 'broke back' (Peters, 1955) in Sierra Leone and also for the circumstance that causes the seeds of *Dichapetalum toxicarium* to be called 'ratsbane'.

APPENDIX

Notes on the Nuclear-Magnetic-Resonance Spectrum of ω-Fluorodecanoic Acid and of a Fluoro-Octadecenoic Acid of Unknown Structure

By N. SHEPPARD

The hydrogen (proton-) resonance spectra of the two compounds were obtained in chloroform solution with a Varian V-4300B spectrometer at 40 Mcyc./sec. Fig. 3 shows those parts of the spectra where resonances are expected to occur for CH bonds adjacent to the electronegative fluorine atom, as in $CH_2F\cdot$ or $CHF\cdot$. The remainder of the spectra were typical of long-chain carboxylic acids.

This part of the spectrum of ω-fluorodecanoic acid is essentially as expected for a [F]—CH_2—[CH_2] group. It consists of a widely spaced pair of bands (separation 48 cyc./sec., centre of gravity σ 0·95) each with triplet contours (fine structure spacing ~6 cyc./sec.), one being sharp and the other blurred. The separation of 48 cyc./sec. is of the size expected for spin–spin splitting of the methylene resonance by the magnetic F nucleus (spin 1/2); the smaller triplet spacing is of the magnitude and type to be anticipated for spin–spin interaction with the second CH_2 group along the chain. The only unusual and unexplained feature of the spectrum is the blurred nature of the triplet at higher field.

The spectrum of the unknown fluoro-octadecenoic acid shows two bands (a triplet at ν 14 cyc./sec. with spacing 6·1 cyc./sec. and a blurred triplet at 61 cyc./sec.) of the same position and appearance as those shown in the first spectrum, with an additional stronger triplet centred at $\nu+1$ cyc./sec. and with a spacing of 3·6 cyc./sec. The first two bands clearly show the presence of the $F\cdot CH_2\cdot CH_2-$ group in the molecule. The third

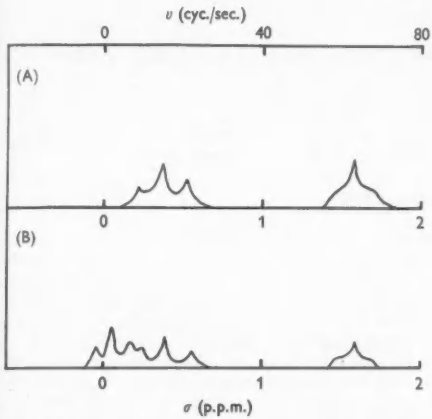

Fig. 3. Nuclear-magnetic-resonance spectra of (*A*) ω-fluorodecanoic acid and (*B*) the unknown fluoro-octadecenoic acid. The spectra are given in frequency units (ν, cyc./sec.) and in chemical-shift units [σ, p.p.m. relative to a water zero defined relative to *cyclo*hexane ($\sigma + 3{\cdot}9$) as an internal standard]. To convert into alternative units (τ, relative to silicon tetramethyl as an internal standard), 4·66 should be added to each value.

additional one is undoubtedly caused by the two ethylenic CH bonds (HC=CH). In this case the triplet structure is caused by spin–spin interaction with adjacent CH_2 groups.

If the fluorine atom on the acid of unknown structure had not been attached to the end of the chain, the groups giving rise to the resonance under discussion would probably have been of the type

$$[CH_2]—CH—[CH_2].$$
$$\quad\quad\quad

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Nitrate Reductase and Related Enzymes in *Escherichia coli*

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Several reports about nitrate reduction in *Escherichia coli* have appeared during the last few years. Taniguchi, Sato & Egami (1956) have found in cell-free extracts of *E. coli*, grown in a peptone broth–agar medium containing nitrate, a particulate system which reduces nitrate to nitrite, only under anaerobic conditions and with reduced diphosphopyridine nucleotide, formate or reduced methylene blue as electron donors and flavin nucleotides as electron carriers. Wainwright (1955) found that nitrate-reductase activity in *E. coli*, strain 1431, was enhanced in anaerobiosis by vitamin K_3 and that the maximal activity was obtained when both vitamin K_3 and flavin nucleotides were added to the incubation mixtures. Cheniae & Evans (1958, 1959) have also observed an activation by vitamin K_3 of nitrate reductase by preparations of *Rhizobium japonicum* when reduced diphosphopyridine nucleotide was the donor. Nicholas & Nason (1955) found in cell-free extracts of *E. coli* grown in a synthetic medium a soluble, flavin-dependent diphosphopyridine nucleotide–nitrate reductase which reduces nitrate to nitrite aerobically. Medina & Heredia (1958) found that cell-free extracts of *E. coli*, grown either in a synthetic medium or in a complex broth medium, contain a reduced diphosphopyridine nucleotide-dependent system which reduces nitrate to nitrite aerobically with vitamin K_3 as electron carrier without any apparent participation of flavin in the process.

The work presented in this paper shows that nitrate reduction takes place under aerobic as well as under anaerobic conditions and in both cases is catalysed by a menadione-reductase–nitrate-reductase system which uses vitamin K_3 as electron carrier. In addition to this pathway, nitrate can also be reduced to nitrite, under completely anaerobic conditions, through an apparently flavin-dependent system, presumably related to the reduced diphosphopyridine nucleotide-oxidase system.

MATERIALS AND METHODS

Preparation of cell-free extracts. Homogenates were prepared from cells of *E. coli*, strain 86 N.C.T.C. (Oxford), grown aerobically in a synthetic medium (Nicholas & Nason, 1955) or in a peptone–beef-extract broth medium containing 0·5% of KNO_3. Round flasks of 3 l. capacity, containing 1·5 l. of medium, were inoculated and allowed to stand at 37° for about 12 hr. Cells were harvested by filtering through Chamberland filters, Pasteur-system type 1 F-NP and centrifuging in the cold at 8000 *g* for 5 min. The cells were washed twice on the centrifuge with 0·9% (w/v) NaCl, and frozen overnight at −15°. The cells were disrupted by grinding in a cold mortar with twice their weight of alumina powder (Alcoa A-301). After grinding for 10 min., four times their weight of 0·1 M-sodium phosphate buffer, pH 7·5, was added, and grinding continued for another 10 min. Homogenates were centrifuged at 8000 *g* for 10 min. in the cold, and the supernatant was used as 'crude extract'. The layer on the top of the alumina, removed and suspended in the above-named buffer, was used as 'large-particle fraction'.

Cofactors. Triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co. The corresponding reduced nucleotides (TPNH and DPNH) were prepared enzymically (Nason & Evans, 1953; Pullman, Colowick & Kaplan, 1952). TPNH and DPNH concentrations were determined spectrophotometrically by using $6{\cdot}22 \times 10^6$ cm.²/mole as extinction coefficient at 340 mμ (Horecker & Kornberg, 1948). Flavin-adenine dinucleotide (FAD) was obtained from Sigma Chemical Co.; flavin mononucleotide (FMN) from Nutritional Biochemicals Corp. and riboflavin from British Drug Houses Ltd. Boiled pig heart was prepared as described by Ochoa (1948). Vitamin K_3 (2-methyl-1:4-naphthaquinone bisulphite) was obtained from the Laboratorios Alter, dicoumarol from the Laboratorios Lefa, Madrid, Spain, and 2-heptyl-4-hydroxyquinoline-*N*-oxide was generously provided by Dr J. W. Cornforth, National Institute for Medical Research, Mill Hill, London.

Determination of enzymic activities. Nitrate-reductase activity was determined by measuring the nitrite formed with a modified sulphanilamide–naphthylamine test (Medina & Nicholas, 1957). Standard conditions were as follows: 0·1 ml. of enzyme was added at zero time to a mixture containing 0·1 ml. of 5 mM-DPNH, 0·1 ml. of 0·1 M-KNO_3, 0·15 ml. of 0·1M-sodium phosphate buffer, pH 7·5, and vitamin K_3 when indicated, to a final volume of 0·6 ml., in open test tubes. After allowing the tubes to stand at 25° for 10 min., the reaction was stopped by adding M-barium acetate (0·1 ml.) and the volume made up to 5 ml. with 95% ethanol. After it was mixed, the suspension was centrifuged at 1500 *g* and tested for nitrite. To 1 ml. of the supernatant were added 1 ml. of sulphanilamide (1% w/v, in 0·1 M-HCl) and 1 ml. of α-naphthylamine hydrochloride (0·02%, w/v). After half an hour, colour intensity was read in a Klett–Summerson photoelectric colorimeter

* Deceased.

Table 1. *Effect of vitamin* K_3 *on nitrate-reductase activity*

Reaction mixtures containing 0·1 ml. of enzyme preparation (as described), 15 μmoles of sodium phosphate buffer, pH 7·5, 10 μmoles of KNO_3, 0·4 μmole of DPNH and additions as indicated, in a final volume of 1 ml., were incubated at 25° for 10 min. Activity is expressed as μm-moles of nitrite produced/10 min./mg. of protein. No nitrite was formed in a control without added DPNH.

	Synthetic medium		Complex broth medium	
Compound added	Crude extract (13·6 mg. of protein/ml.)	Large-particle fraction (33·8 mg. of protein/ml.)	Crude extract (6·9 mg. of protein/ml.)	Large-particle fraction (5·3 mg. of protein/ml.)
—	0	10	180	710
Boiled pig heart	0	10	190	700
FAD (0·1 μmole)	0	10	190	700
Vitamin K_3 (0·1 μmole)	38	20	320	780
Atebrine (2 μmoles)	0	4	95	320
Atebrine (2 μmoles) + FAD (0·4 μmole)	—	4	75	330
Atebrine (2 μmoles) + vitamin K_3 (0·1 μmole)	27	—	360	775
Atebrine (2 μmoles) + vitamin K_3 (0·4 μmole)	57	15	—	—
Dicoumarol (1 μmole)	—	4	—	445
Dicoumarol (1 μmole) + vitamin K_3 (5 μmoles)	—	8	—	505

with the 500 mμ filter. A control without added DPNH was always included. These aerobic conditions were used throughout unless specifically stated otherwise.

Menadione-reductase activity was determined spectrophotometrically, by following DPNH oxidation at 340 mμ (Wosilait & Nason, 1954). Proteins were determined by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

Activation of nitrate reduction by vitamin K_3. Extracts from *E. coli* grown in a synthetic medium or in a complex broth medium with nitrate were assayed for nitrate-reductase activity as described above. No nitrate-reductase activity was detected aerobically in crude extracts of *E. coli* grown in a synthetic medium, unless vitamin K_3 was added (Table 1). The system which was present in the crude extract and in the large-particle fraction was strongly stimulated by vitamin K_3. None of the flavin derivatives tested (FAD, FMN, and riboflavin) showed any activating effect nor did boiled pig heart. Dialysis overnight against 0·1 M-sodium phosphate buffer, pH 7·5, did not decrease the activity of the crude extract as long as the assay for nitrate reduction was carried out with the addition of vitamin K_3.

Atebrine inhibits the system, and the inhibition could not be reversed by FAD but was completely reversed by the addition of a similar amount of vitamin K_3.

The inhibition caused by dicoumarol was also reversed by vitamin K_3.

Aerobic oxidation of reduced diphosphopyridine nucleotide. Table 2 shows the oxidation of DPNH by a crude extract of *E. coli* grown in a synthetic medium. Activity was not affected by nitrate, but it was strongly increased by vitamin K_3. The latter result is explained by the presence in the extracts of menadione reductase (Wosilait & Nason, 1954). In the presence of both vitamin K_3 and nitrate, oxidation of DPNH was accompanied by nitrate reduction. This was not the case when vitamin K_3 was omitted. This suggests that vitamin K_3 can act as electron carrier between DPNH and nitrate.

If purified menadione reductase (Wosilait & Nason, 1954) was substituted for the above-mentioned extract, DPNH oxidation in the presence of vitamin K_3 was not accompanied by nitrate reduction. This observation demonstrates that nitrate reduction involves a different enzyme from the menadione reductase and was not an incidental by-product of the latter.

Inactivation by extraction with light petroleum, and reactivation by the extract and vitamin K_3. Crude

Table 2. *Aerobic oxidation of reduced diphosphopyridine nucleotide by a crude extract of* Escherichia coli

Mixtures containing 0·72 μmole of DPNH, 0·1 ml. of enzyme (crude extract of *E. coli* grown in synthetic medium), 2·5 ml. of 0·1 M-sodium phosphate buffer, pH 7·5, and additions as indicated, in a final volume of 3 ml., were incubated for 10 min. at room temperature (18°). Oxidation of DPNH was followed spectrophotometrically at 340 mμ. After 10 min. samples (1 ml.) were removed and placed in test tubes containing 0·1 ml. of M-barium acetate for determination of the nitrite formed, as described in the Materials and Methods section.

Additions	DPNH oxidized (μm-moles)	Nitrite formed (μm-moles)
None	50	0
KNO_3 (100 μmoles)	50	0
Vitamin K_3 (50 μm-moles)	520	0
Vitamin K_3 (50 μm-moles) + KNO_3 (100 μmoles)	675	225

extracts and the 45 P fraction (see below) from *E. coli* grown in a complex broth medium, which reduced nitrate even without the addition of vitamin K_3, lost a great deal of their nitrate-reductase activity by repeated extractions with light petroleum (b.p. 40–60°). Enzyme preparation was shaken in a test tube with an equal volume of light petroleum for 5 min. at room temperature and centrifuged. Residual nitrate reductase was determined by means of the standard procedure.

These preparations could be reactivated either by vitamin K_3 or by the dry residue obtained by vacuum evaporation of light-petroleum extracts of previously boiled preparations (see Table 3).

Table 3. *Inactivation of nitrate-reductase preparations by extraction with light petroleum and reactivation by the extract and vitamin* K_3

Nitrate-reductase activity was determined with 0·1 ml. of enzyme (*E. coli* grown in complex broth medium) by the standard procedure (see Materials and Methods section) with additions as indicated. Figures represent μm-moles of nitrite formed in 10 min. at 25°. Dried material derived by extracting 20 ml. of boiled enzyme preparation with light petroleum (see text) was suspended in 1·5 ml. of water. In this experiment 0·2 ml. of this suspension was used.

	Additions		
Fractions	None	Light-petroleum extract	Vitamin K_3 (1 μmole)
45 P	57·5	52·5	150
45 P extracted with light petroleum	10	37·5	152

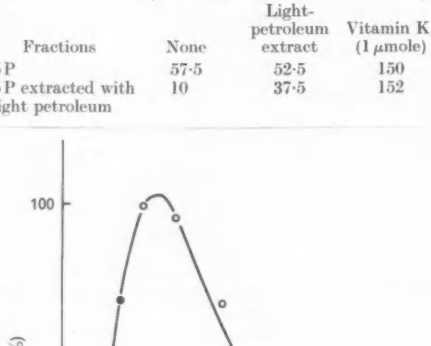


Fig. 1. Effect of pH on nitrate-reductase activity. Mixtures containing 0·5 μmole of DPNH, 10 μmoles of KNO_3, 0·05 μmole of vitamin K_3, 0·3 ml. of 0·1 M-buffer as indicated, and 0·1 ml. of enzyme (45 P fraction from *E. coli* grown in synthetic medium) in a total volume of 0·6 ml. were incubated for 10 min. at 25°. Nitrite was determined as in the standard procedure. The pH was determined in duplicate tubes. Buffers employed were: ●, sodium acetate; ○, sodium phosphate; ▼, glycine. All of these buffers were completely devoid of any inhibitory effect on the enzyme. Activity at pH 5·9 and 25° was 180 μm-moles of nitrite formed/10 min.

Solubility and stability of the system. Nitrate-reductase activity in crude extracts is about one-half of that in homogenates, which suggests that the system is of a particulate nature. This is supported by the fact that almost the whole activity was present in the sediment after centrifuging at 110 000 *g* for 1 hr. Treatment of the crude extracts with ammonium sulphate at 45 % saturation precipitates the system almost completely. This precipitate, suspended in 0·3 vol. of 0·1 M-sodium phosphate buffer, pH 7·5, is referred to as the 45 P fraction.

Nitrate-reductase activity in the crude extracts was rather unstable. Upon storage at −20°, they lost about 75 % of their activity in 1 month. Under the same conditions the 45 P fraction completely maintained its activity. This fraction was less stable after dialysis overnight against 0·1 M-phosphate buffer, pH 7·5.

The nitrate-reductase system completely lost its activity after 5 min. at 70° and pH 7·5.

Effect of pH *upon nitrate-reductase activity.* As shown in Fig. 1, activity was maximal in the range

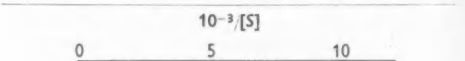


Fig. 2. Effect of nitrate concentration on the rate of nitrate reduction. Mixtures containing 0·9 μmole of DPNH, 0·1 μmole of vitamin K_3, 15 μmoles of sodium phosphate buffer, pH 7·5, 0·1 ml. of enzyme (45 P fraction from *E. coli* grown in synthetic medium) and nitrate as indicated, in a total volume of 0·65 ml., were incubated at 25° for 10 min. Nitrite formed was determined as in the standard procedure. ●, Reaction rate at various concentrations of nitrate; ○, double reciprocal plot (Lineweaver & Burk, 1934).

pH 6–7, with a particularly abrupt decrease below pH 6. Stability is maximal in the range pH 5–8, with a sharp drop above pH 9. Menadione-reductase activity has been reported to be maximal at pH 8·3 with a sharp drop above pH 9 (Wosilait & Nason, 1954).

Effect of concentration of nitrate and vitamin K_3 *on the rate of nitrate reduction.* Nitrate reduction by extracts of *E. coli* under standard conditions with an excess of vitamin K_3 was half maximal at about 0·1 mM-nitrate concentration (Fig. 2). With excess of nitrate, half-maximal activity was obtained at 0·05 mM-vitamin K_3, which is the order of magnitude of the K_m of the menadione reductase (Wosilait & Nason, 1954).

Effect of inhibitors upon the nitrate-reductase–menadione-reductase system. The strong inhibition produced by complexing agents such as cyanide, αα′-dipyridyl and *o*-phenanthroline (Table 4) suggests the participation of some metal constituent in the system. The menadione reductase is not inhibited by complexing agents (Wosilait & Nason, 1954). Participation of a metal constituent in the process is supported by the inactivation produced by dialysis of the enzyme preparations against mM-cyanide.

The strong inhibition produced by 2-heptyl-4-hydroxyquinoline-*N*-oxide seems to take place at the vitamin K_3 level, since it could be reversed by increasing the vitamin K_3 concentration, in a manner suggesting competitive inhibition (Fig. 3).

Table 4. *Effect of inhibitors on the nitrate-reductase–menadione-reductase system*

Mixtures containing 0·1 ml. of crude extract (*E. coli* grown in synthetic medium), 10 μmoles of KNO_3, 15 μmoles of sodium phosphate buffer, pH 7·5, 0·5 μmole of vitamin K_3, 0·5 μmole of DPNH and 0·1 ml. of inhibitor (to give final concentrations as indicated) were incubated in a final volume of 1 ml. Enzyme and inhibitor had been mixed and preincubated for 10 min. at 25° before the addition of the assay reagents. After a further incubation of 10 min., nitrite formed was determined as in the standard procedure.

Compound	Concentration (mM)	Inhibition (%)
KCN	1	100
αα′-Dipyridyl	6	67
o-Phenanthroline	5	90
Sodium diethyldithiocarbamate	1	29
Salicylic acid	1	32
8-Hydroxyquinoline	1	29
Thiourea	1	41
2-Heptyl-4-hydroxyquinoline-*N*-oxide	0·4	95
Urethane	10	0
2:4-Dinitrophenol	0·1	18
Chlortetracycline	0·05	19
Gramicidin	0·02	19

Uncoupling reagents, such as 2:4-dinitrophenol, chlortetracycline and gramicidin, caused little inhibition. The system neither exhibited a phosphate requirement nor was it inhibited by pyrophosphate at concentrations of the order of 0·1 M.

Effect of nitrate on nitrate-reductase formation. Nitrate-reductase activity in crude extracts of *E. coli* grown in either synthetic or complex broth media without added nitrate was about 20 % of that in crude extracts from cells grown with nitrate, indicating that the nitrate reductase is inducible. Menadione-reductase activity was not dependent upon the presence of nitrate in the cultures.

Nitrate reduction in anaerobiosis. Crude extracts and the 45 P fraction from *E. coli* grown in a synthetic medium did not reduce nitrate aerobically unless vitamin K_3 was added but reduced nitrate to nitrite under anaerobic conditions even without addition of vitamin K_3. This anaerobic reduction of nitrate was stimulated by FAD. The 45 P fraction loses this capacity after 5 min. at 60°, whereas its stimulation by vitamin K_3 was resistant to this treatment (Table 5). Menadione-reductase activity was also present after this treatment. This observation is not in agreement with that reported by Wosilait & Nason (1954). Although the difference in results cannot be explained by the available data, it is suggested

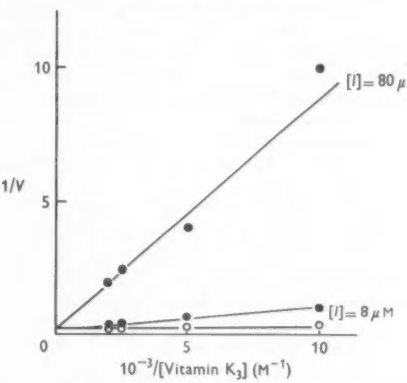


Fig. 3. Competitive inhibition of vitamin K_3 by 2-heptyl-4-hydroxyquinoline *N*-oxide. Representation of Lineweaver & Burk (1934). Mixtures containing 0·5 μmole of DPNH, 10 μmoles of KNO_3, 15 μmoles of sodium phosphate buffer, pH 7·5, 0·1 ml. of enzyme (crude extract of *E. coli* grown in synthetic medium) and vitamin K_3 and inhibitor (*I*) as indicated, in a total volume of 1 ml., were incubated for 10 min. at 25°. Nitrite formed was determined as in the standard procedure. ●, Inhibitor present; ○, inhibitor absent.

Table 5. *Effect of vitamin K_3 and flavinadenine dinucleotide upon the aerobic and anaerobic reduction of nitrate*

Mixtures containing 10 μmoles of KNO_3, 1·3 μmole of DPNH, 15 μmoles of sodium phosphate buffer, pH 7·5, and 0·2 ml. of enzyme (*E. coli* grown in synthetic medium) were incubated for 10 min. at 25°, in a final volume of 0·9 ml. Assays under anaerobic conditions were made in Thunberg tubes. Additions of 0·002 μmole of FAD or 0·2 μmole of vitamin K_3 were made as indicated. Activity is expressed as μm-moles of nitrite formed/mg. of protein.

	Anaerobic				Aerobic			
Fraction	None	FAD	Vitamin K_3	FAD + vitamin K_3	None	FAD	Vitamin K_3	FAD + vitamin K_3
Ppt. from ammonium sulphate at 0–45% saturation	14	35	420	470	0	0	390	390
Ppt. from ammonium sulphate at 0–45% saturation heated for 5 min. at 60°	0	0	320	310	0	0	280	280

that the heat lability found by Wosilait & Nason could be related to the acetone treatment involved in their preparation.

DISCUSSION

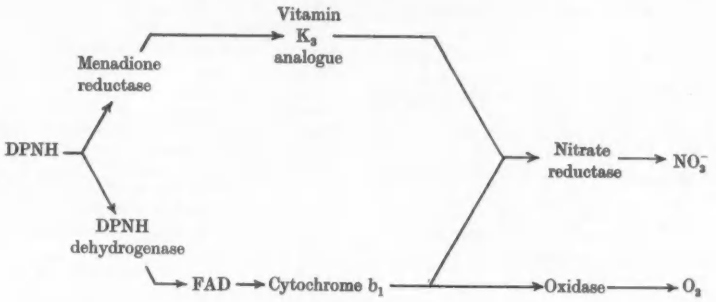
Reduction of nitrate to nitrite in *E. coli* seems to take place through pathways which differ in some of the electron carriers and are detectable depending on the assay conditions. Aerobically, the process is strongly stimulated by addition of vitamin K_3. The presence of a pyridine nucleotide-dependent menadione reductase (Wosilait & Nason, 1954) suggests that, *in vivo*, vitamin K_3 or some related compound acts as electron carrier between DPNH or TPNH and nitrate. This hypothesis is supported by the fact that vitamin K_3 can act at catalytic levels, which suggests that vitamin K_3 is oxidized and reduced alternately. Further support for this contention is provided by the observation that chemically-reduced vitamin K_3 can act as electron donor for the nitrate reductase (A. Medina, unpublished work). The possibility that nitrate could be reduced by reduced vitamin K_3 without participation of a second enzyme is ruled out by the fact that purified menadione reductase is unable to reduce nitrate even in the presence of vitamin K_3.

The strong inhibition produced by complexing agents suggests that some metal is involved in the process. The fact that menadione-reductase activity is not inhibited by these compounds indicates that the metal is involved in the electron transfer from reduced vitamin K_3 to nitrate. The inhibition produced by 2-heptyl-4-hydroxyquinoline-*N*-oxide, which is supposed to be an inhibitor in processes where cytochrome b_1 is involved (Lightbown & Jackson, 1954), seems to be competitive with vitamin K_3.

Crude extracts from *E. coli* grown in a complex broth medium, which reduce nitrate even without any addition of vitamin K_3, can be inactivated by extraction with light petroleum. Reactivation of the residue, by either vitamin K_3 or the ether extract, suggests that the role of vitamin K_3 *in vitro* is accomplished *in vivo* by some lipo-soluble factor, possibly vitamin K_2, which is known to occur in *E. coli* (Baumgärtel & Zahn, 1953).

The aerobic reduction of nitrate was not stimulated by flavins. On the other hand, atebrine, generally considered as typical inhibitor of flavoenzymes, was inhibitory. Nevertheless, the fact that the inhibition by atebrine could be completely reversed by vitamin K_3 (but not by FAD) suggests that no flavoenzyme is involved (Haas, 1944). Nicholas & Nason (1955) have demonstrated the occurrence of protein-bound FAD in a preparation of nitrate reductase from *E. coli*. Nevertheless, the fact that they report an activity in anaerobiosis over twice that in air suggests that their preparation was enriched in DPNH oxidase, which could account for the FAD found (see below).

Anaerobically, nitrate reduction can be stimulated not only by vitamin K_3 but also by FAD. The activity stimulated by FAD can be destroyed by heat-treatment without major impairment of activity stimulated by vitamin K_3. These facts suggest that there are at least two mechanisms through which nitrate can be reduced to nitrite. In one of them, detectable under aerobic as well as in anaerobic conditions, vitamin K_3 or some related compound acts as electron carrier, and, in another one, detectable only under anaerobic conditions, flavin is involved. This fact is in agreement with the finding of Taniguchi *et al.* (1956), who propose that anaerobic nitrate reduction via flavin takes place through an electron-transport chain with some of the electron carriers in common with the DPNH-oxidase chain. Nitrate with terminal nitrate reductase can serve as the terminal



Scheme 1

respiratory acceptor in anaerobiosis, instead of oxygen plus terminal oxidase, which is the normal acceptor in anaerobiosis.

Taniguchi *et al.* (1957) were unable to obtain any stimulating effect by vitamin K_3 on DPNH oxidation. This was presumably due to a loss of the menadione-reductase activity in their preparations. Since the completion of this work, Itagaki & Taniguchi (1959) have reported activation of extracts by vitamin K_3, thus confirming our preliminary report that the latter can be involved in nitrate reduction (Medina & Heredia, 1958).

Our results suggest that the scheme proposed by Taniguchi *et al.* (1957) should be modified to include an alternative pathway which can work in aerobiosis as well as in anaerobiosis. Only the identified steps are included in the minimal Scheme 1.

Although the possibility that the last stage in both processes, the electron transfer from an electron carrier to nitrate, could be catalysed by two different enzymes cannot be excluded, it seems more likely, from specificity considerations, that it is catalysed by a single enzyme. The specific thermal inactivation of the system in which flavin is involved does not necessarily support the participation of two different enzymes, since inactivation could take place in earlier steps of the chain.

The fact that nitrate reduction via flavin seems to take place only under anaerobic conditions suggests that it is intimately linked to the energy metabolism of the cell. In this case nitrate would act as an alternative terminal oxidant, according to the 'nitrate-respiration' theory (Sato, 1950). On the other hand, the fact that the nitrate-reductase system involving vitamin K_3 is oxygen-insensitive and the known ability of *E. coli* to grow on nitrate as the only source of nitrogen, suggests that this system can play a role in nitrogen assimilation. This contention is supported by the observation of the ability of intact *E. coli* to reduce nitrite and hydroxylamine (Taniguchi *et al.* 1956) and the identification in extracts of nitrite- and hydroxylamine-reductase activities (A. Medina, unpublished work).

SUMMARY

1. A system which reduces nitrate to nitrite, with di- or tri-phosphopyridine nucleotide as electron donor and vitamin K_3 as electron carrier, has been found in *Escherichia coli*.

2. The transfer of electrons from pyridine nucleotides to vitamin K_3 is catalysed by the constitutive menadione reductase. Nitrate reductase proper is an inducible enzyme which is associated with particulate matter. Nitrate reductase apparently involves some metal but not flavin.

3. The effects of substrate concentration, pH and other factors have been investigated.

4. Anaerobically, in addition to this pathway, nitrate reduction can take place through a system, apparently flavin-dependent, presumably related to the reduced diphosphopyridine nucleotide oxidase.

5. The possible physiological significance of these two systems able to reduce nitrate is discussed.

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REFERENCES

Baumgärtel, T. & Zahn, D. (1953). *Klin. Wschr.* **31**, 92.
Cheniae, G. M. & Evans, H. J. (1958). *Fed. Proc.* **17**, 201.
Cheniae, G. M. & Evans, H. J. (1959). *Biochim. biophys. Acta*, **35**, 140.
Haas, E. (1944). *J. biol. Chem.* **155**, 321.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Itagaki, E. & Taniguchi, S. (1959). *J. Biochem., Tokyo*, **46**, 1419.

Lightbown, J. W. & Jackson, F. L. (1954). *Biochem. J.* **58**, xlix.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Medina, A. & Heredia, C. F. (1958). *Biochim. biophys. Acta*, **28**, 452.
Medina, A. & Nicholas, D. J. D. (1957). *Biochim. biophys. Acta*, **23**, 440.
Nason, A. & Evans, H. J. (1953). *J. biol. Chem.* **202**, 655.
Nicholas, D. J. D. & Nason, A. (1955). *J. Bact.* **69**, 580.
Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
Pullman, N. E., Colowick, S. P. & Kaplan, N. O. (1952). *J. biol. Chem.* **194**, 593.
Sato, R. (1950). *Scienca Rev.* **2**, 122.
Taniguchi, S., Asano, A., Iida, K., Kono, M., Ohmachi, K. & Egami, F. (1957). *Proc. Int. Symp. enz. Chem., Japan*, p. 238.
Taniguchi, S., Sato, R. & Egami, F. (1956). In *A Symposium on Inorganic Nitrogen Metabolism*, p. 87. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Wainwright, S. D. (1955). *Biochim. biophys. Acta*, **18**, 583.
Wosilait, W. D. & Nason, A. (1954). *J. biol. Chem.* **208**, 785.

Biochem. J. (1960) **77**, 30

The Effect of Growth and Function on the Chemical Composition of Soft Tissues

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During the second half of the nineteenth century a considerable amount of work was done in European countries on the chemical composition of the mammalian body and of its component tissues. The subject then lapsed, and papers on it appeared only occasionally until 10 or 15 years ago, when there was a revival of interest, particularly in the United States. Much of the work has been done on animals, but human tissues have also been studied, generally to see how various diseased states affected their composition.

In spite of all this activity there have, up till now, been very few systematic studies of the changes which take place in tissues with normal development. It has been known for at least 100 years that foetuses and new-born animals contain a higher proportion of water than do adults of the same species (von Bezold, 1857). This is to some extent accounted for by an increasing proportion of fat in the body, but even on a fat-free basis the percentage of water in the whole body falls from embryonic to some time in post-natal life. This percentage then becomes approximately constant at an age which varies from species to species. This 'steady state' was termed by Moulton (1923) 'chemical maturity'. More recently it has been appreciated that the fall in the percentage of water is due largely to a decrease in the proportion of the body occupied by extracellular fluids. Coincident with this there is a rise in the proportion of the body occupied by cells, the cells first increasing primarily in number and later in size.

The body is made up of many different organs and tissues, each with a different chemical composition, and each contributing a different proportion to the body as a whole. The proportion which the various organs contribute to the body, moreover, is not the same at all ages; for example, the brain always accounts for a larger proportion of the body in early life, and skeletal muscle accounts for more of the weight of the adult than of the foetus or new-born baby. The present investigation was designed to study, in man and the pig, the rate at which different parts of the body become chemically mature, and if possible to relate this to the function of these parts at different ages. The effect of development on the chemical composition of the whole bodies of these two species was described some years ago (Widdowson & Spray, 1951; Widdowson, McCance & Spray, 1951; Spray & Widdowson, 1951). More recently the findings on the serum and erythrocytes have been published (Widdowson & McCance, 1956; McCance & Widdowson, 1956*a*) and also those on the skeletal muscle (Dickerson & Widdowson, 1960).

MATERIAL AND METHODS

The sources of the material for this investigation were the same as those for the skeletal muscle described by Dickerson & Widdowson (1960). Skin, heart, liver, kidney and brain have been studied at four or five stages of development in man and five or six stages of development in the pig. The number of samples analysed in each case is given in the tables. Each sample from the foetal and new-born

pigs consisted of pooled material taken from several animals in the same litter; 44 foetal pigs at 46 days of gestation, 22 at 90 days and 12 new-born pigs have been used in the whole investigation.

Skin. This was taken from the abdomen or thigh in new-born and post-natal material and from both these sites in the foetuses. All visible fat was scraped off and the skin was finely cut up with scissors. It included epidermis and corium.

Heart. In some instances whole hearts have been analysed and in others the organ has first been dissected into right and left ventricles and auricles before sampling. All visible fat was removed before dissection and sampling, and the tissue was cut up finely with scissors.

Liver. The organ was blotted free from superficial blood and weighed. The whole of the foetal and new-born livers were taken for analysis and portions of the adult organs were taken from the centre of more than one lobe. The material was homogenized in a Waring Blendor without added water.

Kidney. The capsule was stripped off, and with it the perirenal fat. Both kidneys were taken from the foetuses and new-born babies and piglets, but only one from the adults. The organs were homogenized without added water.

Brain. In every instance the whole brain was taken and again homogenized without added water.

The methods of sampling and all chemical techniques were the same as those described by Dickerson & Widdowson (1960) for skeletal muscle. Fat was determined as the difference in weight before and after extracting the dry solids with light petroleum. Pieces of skin and cardiac muscle taken from the left ventricle were fixed in formalin–0·9% sodium chloride solution (1:9, v/v) for histological examination.

RESULTS

Skin

Tables 1 and 2 show the composition, on a fat-free basis, of human and pig skin at various ages. Development in both species was associated with a fall in the concentration of water, sodium and chloride, and an increase in that of total nitrogen and collagen nitrogen. Collagen nitrogen increased faster than the total nitrogen, and there was therefore an increase in the proportion of the total nitrogen present as collagen. This process had proceeded further in the skin of human babies at birth than in that of new-born piglets. The skin of the infants at 3–6 months was mature in so far as percentages of water and total nitrogen were concerned, but not in the proportion of the total nitrogen contributed by collagen. In the pig, this aspect of development was complete at 6 weeks, although the proportions of total nitrogen, collagen nitrogen and water did not reach a mature level until after this age. The amounts of the cellular constituents, potassium, phosphorus and magnesium, increased during pre-natal growth to a maximal value during early post-natal life, and fell to a lower level in the adult organ. The concentration of calcium in human skin increased during pre-natal growth to a maximum at the end of gestation and decreased during subsequent growth. The results of the analyses of adult human skin are similar to those of Eisele & Eichelberger (1945), and agree with all previous work on the skin of a variety of animals in that they show a higher concentration of chloride than is present in any other tissue except tendon.

Heart

Tables 3 and 4 show the weights and composition of the whole hearts of man and the pig at various stages of development before and after birth.

In man the heart contributed about the same proportion of the body weight at each age, but in the pig the proportion fell. The decrease after birth in the latter species is partly accounted for by the deposition of body fat during post-natal growth.

As in other tissues the proportion of water fell with development and the concentration of nitrogenous constituents rose. High values for calcium were found in the heart muscle of the foetal pig at 46 days of gestation, as they were in foetal skeletal muscle (Dickerson & Widdowson, 1960). There was insufficient material to make the determination in the foetal hearts of man.

The intracellular ion potassium and the extracellular ion sodium behaved in a rather unexpected way. Although the results were rather variable from one heart to another there seemed no doubt that the foetal heart of man generally contained a much higher concentration of potassium and a lower concentration of sodium than did the heart of the baby at term; at 4–7 months the figures were similar to those found at birth, and thereafter the concentrations of the two elements moved in the usual directions. The heart of the pig foetus at 46 days of gestation also contained more potassium and less sodium than did the heart of the new-born animal. The differences were smaller than in human heart, but were none the less statistically significant (potassium, $P < 0{\cdot}01$; sodium, $P < 0{\cdot}01$). In both human and pig heart the concentration of chloride hardly changed throughout development and, if chloride space may be taken as a measure of extracellular fluid, it must be concluded that the heart of the foetus is very near its adult composition in the proportion of it occupied by extracellular fluid; in this respect heart muscle is to be contrasted with skeletal muscle, described by Dickerson & Widdowson (1960).

The heart is a composite structure consisting of two auricles and two ventricles, separated by a strong muscular septum, and valvular, bundle and nodal tissues, and these form different proportions of the whole heart at different ages. It has been shown by other workers that the walls of the different chambers of the heart of an adult man do

Table 1. *Composition of human skin*

Results are expressed per kg. of fresh fat-free skin. Average and range are given.

	Foetus		Baby		
	13–14 weeks	20–22 weeks	New-born	3–6 months	Adult
No. of samples analysed	2	4	4	6	5
Water (g.)	917 (910, 924)	901 (893–910)	828 (800–850)	675 (620–770)	694 (680–710)
Total N (g.)	11·6 (10·1, 13·0)	11·9 (10·9–13·0)	26·5 (22·8–29·2)	54·5 (37·8–69·6)	53·0 (51·0–55·0)
Collagen N (g.)	—	2·4 (1·5–3·7)	16·8 (9·8–21·5)	39·2 (23·2–47·1)	45·7* (45·2, 46·1)
Collagen N (% of total N)	—	20·2 (13·8–28·4)	63·4 (43·0–73·6)	70·5 (61·2–78·4)	89·8* (87·5, 92·0)
Na (m-equiv.)	—	120 (107–130)	87·1 (73·0–104)	69·4 (61·7–76·8)	79·3 (70·5–83·0)
K (m-equiv.)	23·8 (22·0, 25·6)	36·0 (30·0–43·5)	45·0 (40·3–52·0)	43·7 (39·8–49·5)	23·7 (19·2–28·8)
Cl (m-equiv.)	90·6 (86·9, 94·3)	96·0 (91·0–102)	66·9 (63·2–72·0)	72·3 (61·3–82·5)	71·4 (62·0–77·0)
P (m-moles)	41·8 (40·0, 43·6)	28·2 (23·7–31·3)	31·7 (29·4–33·5)	34·9 (31·0–45·3)	14·0 (11·6–17·1)
Mg (m-equiv.)	—	3·8 (1·5–6·0)	4·7 (2·3–7·1)	7·4 (5·3–9·5)	3·1 (1·2–4·3)
Ca (m-equiv.)	4·4 (4·3, 4·4)	6·1 (6·0–6·2)	10·0 (6·5–12·1)	11·4 (10·0–13·4)	9·5 (7·8–12·2)

* Two values only.

Table 2. *Composition of pig skin*

Results are expressed per kg. of fresh fat-free skin. Average and range are given.

	Foetus		Piglet			
	46 days	90 days	New-born	3 weeks	4–6 weeks	Adult
No. of samples analysed	5	2	4	3	3	6
Water (g.)	937 (935–940)	902 (900, 903)	836 (818–852)	782 (755–800)	778 (760–800)	685 (650–710)
Total N (g.)	6·5 (5·7–6·9)	10·6 (10·5, 10·6)	20·4 (18·1–21·7)	36·5 (34·2–41·2)	36·5 (33·3–39·0)	53·2 (47·6–61·0)
Collagen N (g.)	0·4 (all 0·4)	3·6 (3·6, 3·6)	9·6 (8·7–10·1)	18·8 (15·3–23·4)	30·9 (28·4–33·4)	48·5 (44·0–52·5)
Collagen N (% of total N)	6·2 (5·8–7·0)	34·1 (34·0, 34·2)	47·0 (46·6–48·0)	51·5 (44·8–56·8)	84·6 (82·3–89·7)	91·1 (86·0–92·4)
Na (m-equiv.)	127 (115–152)	96·0 (94·0, 98·0)	110 (100–127)	90·2 (79·0–100)	86·8 (86·0–88·0)	81·6 (68·2–105)
K (m-equiv.)	28·3 (24·3–32·9)	39·5 (39·2, 39·8)	39·5 (34·0–44·5)	44·5 (39·7–47·2)	41·9 (39·8–43·0)	26·2 (17·8–36·5)
Cl (m-equiv.)	93·7 (86·5–97·5)	81·0 (79·2, 81·7)	80·0 (78·0–84·5)	74·7 (67·5–79·0)	75·5 (70·0–81·0)	74·4 (66·0–83·5)
P (m-moles)	15·8 (12·6–17·4)	28·6 (28·2, 28·9)	31·2 (26·2–39·5)	38·8 (33·8–47·5)	34·4 (31·8–35·8)	17·2 (12·3–22·7)
Mg (m-equiv.)	4·3 (3·0–5·5)	7·2 (6·7, 7·7)	6·6 (5·7–8·1)	7·8 (6·3–9·0)	7·8 (7·3–8·1)	4·4 (3·0–5·8)
Ca (m-equiv.)	10·2 (9·2–11·4)	11·3*	9·1 (6·1–11·2)	10·8 (8·3–12·7)	8·4 (7·3–10·0)	9·2 (6·5–12·4)

* One result only.

Table 3. *Composition of human heart*

Results are expressed per kg. of fresh fat-free heart. Average and range are given.

	Foetus	Baby		
	20–22 weeks	New-born	5–7 months	Adult
No. of hearts analysed	4	4	2	2
Wt. of heart (g.)	1·6 (1·2–2·2)	17 (10–22)	40 (37, 43)	230 (216, 245)
Wt. of heart as % of body wt.	0·63 (0·57–0·71)	0·63 (0·40–0·75)	0·50 (0·47, 0·53)	0·42 (0·38, 0·46)
Water (g.)	860 (837–879)	841 (820–857)	830 (825, 835)	827 (825, 828)
Total N (g.)	14·0 (12·3–15·2)	19·6 (18·0–22·6)	21·0 (20·2, 21·8)	22·9 (22·7, 23·1)
Non-protein N (g.)	1·5 (1·3–1·6)	1·7 (1·5–1·9)	1·7 (1·6, 1·8)	2·2 (1·8, 2·6)
Collagen N (g.)	0·8 (0·7–0·9)	2·0 (1·5–2·4)	3·5 (3·4, 3·6)	2·4 (1·8, 3·0)
Na (m-equiv.)	46·1 (37·7–58·5)	64·2 (60·2–66·7)	59·8 (57·5, 62·0)	57·8 (57·8, 57·8)
K (m-equiv.)	81·1 (56·7–101·5)	54·3 (37·6–62·5)	49·3 (46·9, 51·6)	66·5 (65·5, 67·5)
Cl (m-equiv.)	41·0 (36·1–46·0)	45·2 (41·2–49·0)	49·3 (49·2, 49·4)	45·6 (40·0, 51·2)
P (m-moles)	49·7 (30·0–63·2)	47·0 (42·2–54·2)	49·5 (48·5, 50·5)	49·0 (47·2–50·8)
Mg (m-equiv.)	—	10·9 (8·9–12·5)	11·0 (10·4, 11·6)	13·2 (11·7, 14·6)
Ca (m-equiv.)	—	7·4 (5·3–8·3)	8·2 (7·1, 9·3)	— (5·5, 12·6)

Table 4. *Composition of pig heart*

Results are expressed per kg. of fresh fat-free heart. Average and range are given.

	Foetus		Piglet			
	46 days	90 days	New-born	3 weeks	4 weeks	Adult
No. of hearts analysed	5 litters	2 litters	6	2	2	2
Wt. of heart (g.)	0·31 (0·18–0·47)	3·5*	8·4 (6·7–10·3)	20 (15, 25)	40 (31, 49)	420 (410, 430)
Wt. of heart as % of body wt.	1·24 (1·03–1·46)	0·55*	0·71 (0·66–0·76)	0·57 (0·40, 0·73)	0·56 (0·45, 0·67)	0·21 (0·21, 0·21)
Water (g.)	876 (857–885)	868 (865, 870)	830 (817–858)	825 (815, 835)	820 (818, 822)	825 (815, 835)
Total N (g.)	15·1 (14·0–17·0)	16·3 (16·2, 16·3)	20·8 (18·4–22·0)	22·9 (22·6, 23·2)	23·8 (22·8, 24·8)	26·2 (24·4, 28·0)
Non-protein N (g.)	1·3 (1·2–1·5)	2·0 (1·9, 2·1)	1·9 (1·7–2·0)	2·1 (1·9, 2·2)	2·3 (2·2, 2·4)	2·5 (2·3, 2·7)
Collagen N (g.)	0·35 (0·32–0·38)	0·61 (0·60, 0·62)	0·81 (0·65–0·98)	0·60 (0·41, 0·80)	0·99 (0·95, 1·03)	1·7 (1·7, 1·7)
Na (m-equiv.)	46·0 (33·1–55·0)	52·8 (52·7, 52·9)	56·8 (45·7–69·5)	45·7 (44·4, 47·0)	43·5 (41·5, 45·5)	45·8 (45·0, 46·6)
K (m-equiv.)	93·9 (89·0–96·8)	75·4 (71·8, 79·0)	82·2 (74·3–94·0)	89·5 (82·5, 96·5)	100 (97·0, 103)	87·5 (86·3, 88·7)
Cl (m-equiv.)	36·3 (34·4–38·1)	38·4 (36·9, 39·8)	36·6 (29·2–44·8)	34·3 (33·5, 35·1)	36·0 (32·0, 40·0)	32·0 (29·7, 34·2)
P (m-moles)	46·8 (33·0–57·0)	53·1 (52·3, 53·8)	69·0 (62·5–75·0)	75·8 (67·6, 84·0)	76·0 (74·0, 78·0)	68·2 (66·4, 70·0)
Mg (m-equiv.)	14·2 (13·6–14·7)	10·5 (10·1, 10·8)	16·0 (14·5–18·4)	16·2 (14·0, 18·3)	18·7 (17·6, 19·8)	19·2 (17·2, 21·2)
Ca (m-equiv.)	13·5 (12·0–14·7)	5·9 (5·4, 6·3)	6·3 (3·9–8·2)	5·8 (5·4, 6·2)	2·9 (2·8, 3·0)	3·3 (3·1, 3·5)

* Hearts of only one litter weighed.

Table 5. *Composition of ventricles and auricles of human heart*

Results are expressed per kg. fat-free tissue solids. Average and range are given.

	Right ventricle				Left ventricle				Auricles			
	Foetus 20–22 weeks	Baby		Adult	Foetus 20–22 weeks	Baby		Adult	Foetus 20–22 weeks	Baby		Adult
		New-born	5–7 months			New-born	5–7 months			New-born	5–7 months	
No. of hearts analysed	3*	7	2	2	3*	5	2	7	3*	7	2	2
Total N (g.)	116	128 (118–143)	121 (112, 129)	127 (121, 132)	111	115 (98–134)	123 (111, 135)	127 (121–129)	130	134 (113–143)	127 (125, 129)	135 (129, 140)
Na (m-equiv.)	314	408 (362–454)	364 (344, 384)	—	392	373 (328–454)	375 (356, 394)	253 (228–299)	422	371 (342–400)	343 (328, 358)	—
K (m-equiv.)	435	301 (264–365)	290 (270, 309)	341 (317, 365)	505	292 (244–325)	309 (281, 336)	404 (362–448)	410	243 (222–278)	248 (232, 264)	298 (276, 320)
Cl (m-equiv.)	323	310 (278–391)	308 (304, 312)	339 (292, 386)	305	265 (194–314)	282 (268, 296)	201 (152–236)	—	271 (229–349)	278 (271, 285)	334 (320, 348)
P (m-moles)	365	309 (278–357)	290 (280, 300)	265 (264, 266)	352	307 (280–354)	323 (323, 323)	295 (264–319)	318	242 (186–278)	216 (202, 229)	192 (189, 195)

* One pooled sample was analysed.

not have exactly the same chemical composition (Cullen, Wilkins & Harrison, 1933; Wilkins & Cullen, 1933; Mangun, Reichle & Myers. 1941; Clarke & Mosher, 1952). For example, the auricles contain most water, sodium and chloride, and least potassium and phosphorus; the left ventricle contains least water, sodium and chloride, and most potassium and phosphorus; the right ventricle contains intermediate amounts of all constituents. In ox heart the nodal tissues and tricuspid valve have been shown to contain considerably more sodium and less potassium than the auricles (Davies, Davies, Francis & Whittam, 1952). Changes in composition of the heart, as a whole, are therefore the result of changes in the composition of the different parts, and of the contribution of these different parts to the whole.

In order to obtain more information about the unexpected decrease in potassium and increase in sodium which had been found to take place in the whole hearts of man between 20 weeks of gestation and birth, a further series of hearts was collected, and these were dissected into right and left ventricles and auricles and analysed. The different parts of the hearts, particularly those of the individual foetuses, had to be stored for some time at $-20°$ before analysis and it was evident from the values obtained for the various constituents that a variable amount of drying had taken place during storage. For this reason, the composition of the different parts of the heart has been expressed per kg. of dry fat-free solids. Table 5 shows the results. Again, the decrease in concentration of potassium during the latter part of gestation is seen, and it is evident that all the chambers participate. Two possible explanations for this are suggested below.

The results in Table 5 for the different chambers of adult heart agree with the previous results of the workers cited above. For most constituents there was a smaller difference between the composition of right and left ventricles in the immature hearts than there was in the adult organs.

Liver

Tables 6 and 7 show the gross composition of the liver at various ages in man and the pig. There are certain features of these results common to both species, and other features in which the two species differ. The general increase in weight of the liver with development is part of the process of growth, and in other species it is known to be accompanied by an increase in the number and size of the cells (Kennedy & Pearce, 1958). The liver formed a more uniform percentage of the body weight in man than in the pig over the age range studied. Had younger human foetuses been available larger livers in proportion to the body weight would no doubt have been found (Hamilton, Boyd & Mossman, 1945). The

Table 6. *Composition of human liver*

Results are expressed per kg. of fresh liver. Average and range are given.

	Foetus		Baby		
	13–14 weeks	20–22 weeks	New-born	4–7 months	Adult
No. of livers analysed	2	4	4	3	4
Wt. of liver (g.)	1·18 (1·10, 1·26)	10·7 (9·2–12·3)	125 (120–130)	310 (290–325)	1570 (1335–2035)
Wt. of liver as % of body wt.	4·0 (3·9, 4·1)	4·2 (3·7–4·9)	4·6 (3·7–5·2)	4·0 (3·6–4·4)	2·4 (2·2–2·7)
Water (g.)	849 (826, 871)	812 (792–825)	786 (780–798)	764 (761–767)	711 (636–739)
Total N (g.)	20·2 (17·4, 23·1)	22·1 (20·3–22·7)	22·6 (20·1–26·7)	24·4 (25·1–26·3)	28·2 (27·8–28·9)
Na (m-equiv.)	—	54·8 (49·5–59·7)	59·8 (58·6–61·9)	51·0 (48·0–56·2)	42·5 (39·8–50·0)
K (m-equiv.)	81·8 (78·5, 85·0)	92·9 (85·0–100)	58·7 (50·7–68·2)	66·2 (64·5–67·0)	75·0 (71·5–82·5)
Cl (m-equiv.)	62·2 (61·3, 63·0)	57·1 (50·5–61·8)	55·8 (53·0–59·0)	42·8 (40·5–44·3)	38·3 (30·0–47·6)
P (m-moles)	82·5 (73·0, 92·0)	88·0 (79·6–102)	56·5 (47·5–71·0)	82·5 (81·0–84·2)	86·0 (74·3–95·0)
Mg (m-equiv.)	—	14·7 (13·6–16·8)	10·4 (9·2–12·4)	11·8 (11·7–12·2)	15·2 (13·5–18·0)
Ca (m-equiv.)	—	2·3 (1·9–2·9)	3·0 (2·3–4·2)	4·4 (3·8–5·0)	2·8 (2·6–2·9)

Table 7. *Composition of pig liver*

Results are expressed per kg. of fresh liver. Average and range are given.

	Foetus		Piglet			
	46 days	90 days	New-born	3 weeks	4 weeks	Adult
No. of samples analysed	5	1	6	1	2	5
Average wt. of liver (g.)	2·2 (1·3–3·5)	15·5	37 (20–54)	92	198 (190, 206)	2570 (2246–3102)
Wt. of liver as % of body wt.	9·4 (7·8–10·8)	2·5	3·0 (2·2–3·4)	2·7	2·8 (2·6, 3·0)	1·3 (1·1–1·6)
Water (g.)	822 (813–833)	816	784 (759–806)	759	746 (744, 747)	716 (708–724)
Total N (g.)	22·9 (19·7–24·8)	21·8	20·2 (19·4–24·8)	27·4	26·8 (26·7, 26·8)	34·5 (33·2–36·7)
Protein N (g.)	20·2	18·5	17·4	23·8	23·3	30·6
Na (m-equiv.)	59·3 (45·0–74·2)	63·6	50·7 (38·4–62·3)	51·0	34·4 (31·4, 37·4)	35·8 (32·6–40·9)
K (m-equiv.)	74·6 (61·8–86·0)	64·0	85·1 (67·0–95·0)	71·6	87·1 (84·0, 90·2)	82·2 (78·3–84·5)
Cl (m-equiv.)	51·4 (45·2–61·0)	56·2	39·7 (29·8–49·0)	32·4	29·0 (27·8, 30·2)	29·2 (27·1–31·6)
P (m-moles)	100 (91–108)	78	82 (72–94)	103	116 (115, 117)	119 (117–123)
Mg (m-equiv.)	19·0 (14·8–21·1)	16·2	16·8 (12·9–20·6)	18·7	18·1 (18·0, 18·2)	19·4 (18·9–20·9)
Ca (m-equiv.)	3·4 (2·3–4·3)	4·2	2·2 (1·9–2·5)	2·6	2·0 (1·8, 2·1)	2·6 (2·2–2·9)

small percentage of the body occupied by the liver in adult pigs is due, in part, to the large amount of body fat in these animals.

There was a steady fall in the proportion of water and a rise in the proportion of nitrogen in both species, broken for a short period at birth in piglets, probably owing to the large amount of glycogen accumulating in the organ at that time (McCance & Widdowson, 1959). The livers of the human foetuses, like the hearts, contained quite remarkably large amounts of potassium and there is no obvious explanation unless it was associated

Table 8 *Composition of human kidneys*

Results are expressed per kg. of fresh whole kidney. Average and range are given.

	Foetus		New-born baby	Adult
	13–14 weeks	20–22 weeks		
No. of samples analysed	2	4	4	4
Average wt. of two kidneys (g.)	0·20 (0·18, 0·22)	1·73 (1·41–1·89)	28 (24–32)	340 (213–452)
Wt. of kidneys as % of body wt.	0·64 (0·60, 0·68)	0·68 (0·60–0·74)	1·02 (0·91–1·16)	0·52 (0·35–0·60)
Water (g.)	915 (914, 915)	884 (876–891)	841 (825–853)	810 (795–821)
Total N (g.)	12·5 (11·9, 13·0)	14·2 (11·4–15·7)	19·2 (17·6–21·1)	24·5 (21·3–27·6)
Na (m-equiv.)	—	68·0 (57·0–73·5)	75·5 (70·7–80·3)	82·0 (79·0–84·4)
K (m-equiv.)	—	66·5 (59·5–78·5)	56·0 (50·5–61·0)	57·0 (52·5–59·7)
Cl (m-equiv.)	66·6 (65·4, 67·8)	59·6 (55·5–66·0)	56·4 (49·5–60·0)	67·8 (59·8–73·0)
P (m-moles)	72·0 (71·0, 73·0)	65·5 (60·0–69·0)	61·0 (57·5–66·0)	57·5 (49·0–66·0)
Mg (m-equiv.)	5·3 (5·3, 5·3)	8·6 (6·5–12·4)	8·7 (6·6–11·5)	8·6 (7·1–9·7)
Ca (m-equiv.)	18·4 (18·0, 18·8)	17·4 (14·7–19·5)	7·7 (5·1–9·9)	7·0 (5·6–8·0)

Table 9. *Composition of pig kidneys*

Results are expressed per kg. of fresh whole kidney. Average and range are given.

	Foetus		Piglet			
	46 days	90 days	New-born	3 weeks	4 weeks	Adult
No. of samples analysed	5	2	4	2	2	5
Average wt. of two kidneys (g.)	0·45 (0·21–0·89)	4·6*	9·8 (7·7–11·9)	25 (22, 28)	38 (32, 43)	436 (396–474)
Wt. of kidneys as % of body wt.	1·8 (1·5–2·0)	0·70*	0·82 (0·70–0·93)	0·70 (0·63, 0·76)	0·54 (0·47, 0·60)	0·23 (0·22–0·23)
Water (g.)	889 (878–927)	874 (873, 875)	825 (790–860)	811 (811, 811)	808 (802, 814)	812 (801–824)
Total N (g.)	13·6 (13·4–14·6)	14·3 (14·3, 14·4)	20·3 (18·0–21·5)	21·5 (20·8, 22·1)	22·0 (21·7, 22·3)	23·7 (22·7–24·4)
Na (m-equiv.)	96·2 (78·5–115)	78·3 (76·5, 80·1)	76·0 (57·5–105)	71·4 (70·0, 72·8)	57·8 (57·6, 58·0)	62·9 (48·7–73·3)
K (m-equiv.)	48·6 (45·0–52·8)	54·5 (52·6, 56·4)	77·4 (60·0–97·5)	67·9 (62·6, 73·2)	76·5 (65·0, 88·0)	60·1 (50·5–68·5)
Cl (m-equiv.)	61·9 (59·5–65·8)	59·1 (57·3, 60·8)	54·9 (49·2–59·0)	59·6 (57·3, 61·8)	48·0 (44·8, 51·2)	57·0 (52·2–60·5)
P (m-moles)	63·0 (60·0–69·0)	54·8 (54·0, 55·5)	84·0 (64·6–108)	81·6 (71·6, 91·6)	89·0 (84·0, 94·0)	69·0 (62·5–75·0)
Mg (m-equiv.)	8·8 (8·5–9·1)	11·2 (11·0, 11·3)	13·2 (10·9–15·6)	13·8 (12·1, 15·5)	13·7 (12·2, 15·2)	8·9 (7·2–10·3)
Ca (m-equiv.)	7·9 (6·8–9·0)	5·0 (4·4, 5·5)	4·6 (4·5–4·7)	9·3 (8·3, 10·3)	4·4 (3·5, 5·4)	4·4 (3·2–5·5)

* Kidneys from only one litter were weighed.

with the haemopoietic activity of the organ before birth (Hamilton *et al.* 1945). The concentration of sodium was not correspondingly low, and the sum of the sodium and potassium was higher than at any other age. The values for sodium, potassium and chloride in the livers of the foetal and new-born pigs showed rather wide variations from one sample to another. Active formation of glycogen is probably one reason for the high values for potassium in the liver of the new-born pig (McCance & Widdowson, 1959). Over the whole age range the protein nitrogen/potassium ratio increased in both species. This has been attributed to a decrease in the amount of water in the cells (McCance & Widdowson, 1956*b*); there is less than 1% of collagen in human liver (Lowry, Gilligan & Katersky, 1941), so that most of the nitrogen must be in the cells, but if haemopoietic tissue has a lower nitrogen/potassium ratio than true liver, this would account for the change. Human liver contained appreciably less phosphorus and magnesium than pig liver at comparable stages of development. High values for calcium, similar to those found in foetal skeletal muscle and heart, were not found in foetal liver.

The immature livers of both species contained more sodium and chloride than the adult organs, suggesting that they contained more extracellular fluid, but the possibility that the cells contained sodium and chloride cannot be ruled out (Yannet & Darrow, 1940). It has not been deemed wise to make calculations of intracellular water on the basis of chloride space.

Kidney

Tables 8 and 9 show the weight and composition of the kidneys. In man the kidneys at birth formed a higher proportion of the body weight than they did at any other age, but in the pig the kidneys contributed most to the total weight of the body at 46 days of gestation. At this stage of the pig's existence the mesonephros, which has been a large functional organ (Stanier, 1960), is shrinking rapidly and the metanephros is growing very fast; 2 days earlier (at 44 days of gestation) the metanephros formed about the same proportion of the body weight as it did at 46 days, and the mesonephros comprised another 1%.

As in other soft tissues, chemical development of the kidneys involved a decrease in the proportion of water and an increase in the proportion of nitrogen. The figures for inorganic constituents were rather erratic, owing no doubt to the presence of fluids filtered off or secreted by the organ, even in the earliest stages. The high values for calcium in the foetal kidneys should perhaps be noted, though at present they remain unexplained. High concentrations of calcium have been reported in foetal membranes (Economou-Mavrou & McCance, 1958), as well as in foetal skeletal muscle (Dickerson & Widdowson, 1960) and heart (see above).

Brain

The weights and gross chemical composition of the brains of the two species are shown in Tables 10 and 11. At all ages the brain of man formed a much larger proportion of the body than did the brain of the pig, and both in the new-born and in the adult the human brain weighed more than ten times as much as the brain of the pig.

The changes in composition in the two species followed the same course, the proportion of water falling from over 90% in the foetus to 76–77% in the adult. The proportion of nitrogen increased, but was always higher at the selected stages of development in the pig than in man, and the proportion of water was correspondingly lower. The concentration of phosphorus was higher in the pig at all ages. In both species the concentration of sodium and chloride decreased with development and the concentration of potassium increased; the big change towards the adult level had already occurred by the time of birth in the pig, but did not occur until after birth in man. Figures for calcium in the pig brains have not been included because some of the adult skulls were sawn in half before the brain was removed, and the values for calcium suggested that the brain samples were contaminated with bone dust.

DISCUSSION

The form and structure of the adult organism is reached by a process of differential growth. The chemical development of the organism is also a differential process, for from the results described above, and from those of Dickerson & Widdowson (1960) for skeletal muscle, there is no doubt that some organs and tissues mature faster than others. Foetal heart muscle and foetal liver and kidneys are much nearer the chemical composition of the adult organs than are skeletal muscle or skin. It is tempting to relate these differences in the rate of chemical development with variations in the speed of functional maturation. The heart sounds can usually be heard at 18–20 weeks of gestation in man, and in the pig the heart has begun to beat before the twenty-first day of gestation, when the animal still weighs only 0·2 g., and Stubbs & Widdas (1959) have shown that contraction of the heart muscle is associated with extrusion of interstitial fluid. The liver functions actively as a haemopoietic organ during gestation and the kidneys certainly produce a urine which is usually, if not always, hypo-osmotic with respect to sodium and chloride. In contrast to these organs, the voluntary

muscles and skin of the foetus have little or no work to do. Observations made on perfused non-viable human foetuses have suggested that the foetus may be quite active *in utero* (Westin, Nyberg & Enhörning, 1958), but even so the work done by the skeletal muscles must be extremely small compared with that done by the beating heart. In the skeletal muscle of the foetus the cells are small and are widely separated by extracellular material (Dickerson & Widdowson, 1960). In foetal heart muscle the cells are also small, but they are packed closely together and there is no space for the large amounts of extracellular material found in skeletal muscle.

Table 10. *Composition of human brain*

Results are expressed per kg. of fresh whole brain. Average and range are given.

	Foetus		New-born baby	Adult
	13–14 weeks	20–22 weeks		
No. of brains analysed	2	4	4	4
Wt. of brain (g.)	4·65 (4·42, 4·88)	34 (25–41)	365 (330–430)	1438 (1250–1640)
Wt. of brain as % of body wt.	15·0 (15·0, 15·0)	13·4 (12·5–14·5)	13·4 (12·2–15·1)	2·3 (2·1–2·8)
Water (g.)	914 (911, 916)	922 (911–929)	897 (891–903)	774 (763–785)
Total N (g.)	9·6 (9·4, 9·7)	8·4 (7·2–9·5)	9·3 (9·0–9·9)	17·1 (16·2–17·7)
Na (m-equiv.)	97·5 (91·0, 102)	91·7 (83·8–98·1)	80·9 (78·5–85·7)	55·2 (54·0–57·1)
K (m-equiv.)	49·6 (44·2, 55·0)	52·0 (47·8–60·0)	58·2 (55·0–60·0)	84·6 (74·0–90·9)
Cl (m-equiv.)	72·1 (68·0, 76·2)	72·6 (69·0–76·2)	66·1 (64·5–67·7)	40·5 (38·1–42·8)
P (m-moles)	57·0 (56·5, 57·5)	52·2 (45·5–58·5)	54·0 (51·2–57·5)	109 (101–115)
Mg (m-equiv.)	—	8·4 (7·8–8·9)	7·9 (7·3–8·3)	11·4 (10·9–11·7)
Ca (m-equiv.)	—	4·9 (3·9–6·0)	4·8 (4·4–5·0)	4·0 (3·0–5·5)

Table 11. *Composition of pig brain*

Results are expressed per kg. of fresh whole brain. Average and range are given.

	Foetus	Piglet			
	46 days	New-born	3 weeks	4 weeks	Adult
No. of brains analysed	5	4	2	2	5
Wt. of brain (g.)	0·87 (0·44–1·28)	33 (32–34)	44 (42, 45)	45 (41, 50)	100 (83–117)
Wt. of brain as % of body wt.	3·5 (2·8–4·2)	2·8 (2·4–3·4)	1·2 (1·1, 1·3)	0·6 (0·6, 0·7)	0·05 (0·04–0·06)
Water (g.)	908 (901–915)	847 (842–859)	820 (815, 825)	812 (805, 819)	758 (744–775)
Total N (g.)	10·0 (8·8–10·4)	14·2 (13·2–15·6)	14·3 (14·0, 14·6)	15·3 (15·0, 15·5)	19·1 (16·9–23·0)
Na (m-equiv.)	82·8 (74·4–85·2)	59·9 (52·6–75·2)	64·1 (61·0, 67·1)	61·4 (55·5, 67·2)	60·7 (50·5–67·9)
K (m-equiv.)	56·7 (54·3–58·0)	85·8 (80·5–89·0)	64·6 (57·0, 72·1)	75·9 (63·8, 88·0)	76·0 (66·6–83·0)
Cl (m-equiv.)	62·3 (59·5–65·6)	47·8 (47·0–49·0)	48·9 (44·5, 53·2)	45·2 (45·0, 45·3)	41·1 (36·4–45·3)
P (m-moles)	57·0 (50·0–64·0)	82·5 (80·5–84·0)	82·0 (80·5, 83·5)	92·5 (92·0, 93·0)	125 (122–130)
Mg (m-equiv.)	9·5 (9·2–9·8)	10·6 (9·9–11·8)	9·2*	8·0 (7·7, 8·2)	12·2 (10·2–13·9)

* One result only.

The changes in the electrolyte composition of the heart and of its several compartments between 20 weeks of gestation and the time of birth in man were rather puzzling, for the high concentration of sodium and low concentration of potassium at term as compared with the foetal heart at 20 weeks of gestation suggested that the whole process of development had been reversed, but, on the other hand, the proportions of water and nitrogen indicated that chemical development had proceeded in the expected direction.

There seem to be two possible ways in which this could have been brought about. Foetal heart muscle may contain some organic anion, for example a protein, peculiar to this time of life, which binds potassium. It will be recalled that the liver of the human foetus also contained more potassium than did the liver of the full-term baby. The high concentration of potassium in the foetal heart could hardly have been due to the formation of glycogen, for the hearts of two normal 20-week foetuses, obtained immediately after delivery, contained only 1·16 and 0·35 g. of glycogen/100 g. There may, however, be some other substance which has not as yet been identified.

Alternatively, it may be that the heart-muscle cells of the full-term babies studied in this investigation had become permeable to sodium, and a corresponding amount of potassium had left them. This is what happens when strips of rat ventricle are suspended in a physiological solution, and the supply of oxygen is cut off (Hercus, McDowall & Mendel, 1955; McDowall, Munro & Zayat, 1955); it has also been shown to take place in man in recently infarcted myocardium (Alexander, Boyle, Iseri, McCaughey & Myers, 1950). The still-born babies, whose hearts were analysed, probably died from a lack of oxygen, and the same thing is true of the older babies who were suffocated in their cots. Their hearts had undoubtedly gone on beating until the time of death, their failure indeed had probably been the cause of death, and it is possible that the anoxia had prevented the cells extruding sodium in the normal way, so that an exchange of sodium and potassium had taken place through the cell walls. Calculations of intracellular sodium, based on the chloride space as a measure of extracellular fluid, showed that the cells of the heart at birth contained more sodium than at any other age examined. Babies suffering from respiratory distress have been shown to have high concentrations of potassium in the serum (Usher, 1959), and this potassium may have come in part from the heart-muscle cells. These babies, moreover, had electrocardiographic abnormalities. It is at present impossible to decide between these two alternatives, and it may even be that both are involved. The matter is under further investigation.

The values obtained for the various constituents in the brain of the adult of the two species are in agreement with those collected by Rossiter (1955), from the literature, for the composition of mammalian brain. It seems certain that, at all stages of development, human brain contains a higher proportion of water than pig brain. The explanation for this may be that grey matter forms a greater percentage of the brain substance in man than in the pig, and grey matter is known to contain a higher proportion of water than white matter (Stewart-Wallace, 1939).

Kerpel-Fronius (1937) showed that the concentration of chloride in the brain of man, the dog and the rabbit fell during post-natal development, and Flexner & Flexner (1950) found that the proportion of extracellular fluid, measured as the chloride space, in the cerebral cortex of the guinea pig decreased steadily from the forty-second day of gestation to term (sixty-sixth day); in the newborn it had the same value as it had in the adult. The chloride space in human brain at the younger ages was found to be greater than it was in the pig at corresponding stages of development. This may mean that human brain contains a greater proportion of extracellular fluid and the same conclusion might be drawn from measurements of the sodium spaces. It has, however, been stated that the minute chemical structure of the brain is so complex that it would be 'unreasonable to interpret' the chemical results in terms of a simple mixture of extracellular and intracellular phases (Manery & Hastings, 1939). There is also evidence from perfusion experiments that much of the chloride of the brain, of some species at any rate, is intracellular (Amberson, Nash, Mulder & Binns, 1938; Oster & Amberson, 1939). Measurements *in vivo* of the volume of distribution in brain of inulin, iodide, *p*-aminohippuric acid and sucrose have given results of less than 5% of the wet weight (Davson & Spaziani, 1959; Morrison, 1959).

Whatever the real distribution of the chloride ion may be, it seems certain that at all stages of development, in both man and the pig, the volume of distribution of the sodium ion is greater, so that some of the sodium is certainly in the cells. Flexner & Flexner (1949) found that the influx of sodium into the cells of the guinea-pig's cerebral cortex, as calculated from the sodium and chloride spaces, coincided with the onset of electrical activity.

The extracellular phase of any tissue may be considered to consist of extracellular water and the connective-tissue proteins (Manery, Danielson & Hastings, 1938). Some of the extracellular water is associated with the fibrous connective-tissue proteins, collagen, elastin and reticulin, to constitute the dense fibrous connective tissue, and this part

of the extracellular water may be referred to as 'fibre' water. The rest of the water outside the cells is associated with the non-fibrous material making up the connective tissue, e.g. mucopolysaccharides and plasma proteins. This concept of the extracellular phase is of importance in relation to the composition of the skin because of the large amount of dense, fibrous, connective tissue which is present in the adult organ.

The partition of the extracellular water between the fibrous and non-fibrous connective-tissue proteins in the skin can be calculated from the amount of collagen in the tissue if it is assumed that the composition of the dense fibrous connective tissue is the same as tendon. A few samples of pig tendon were therefore analysed for water, total nitrogen, collagen and chloride. The values obtained for these constituents were similar to those given by Eichelberger, Eisele & Wertzler (1943) for dog tendon. On the assumption that all the water in tendon is associated with fibrous connective-tissue protein, the results of the analyses of pig tendon have been used to calculate the amount of water associated with the collagen fibres ('fibre' water) in the skin of the pig and man at the different stages of development. The amount of chloride present in this 'fibre' water, at the same concentration as in tendon water, has then been subtracted from the total chloride in the tissue to obtain the amount of this ion present in the 'non-fibre' water, the volume of which has been calculated in the usual way from the concentration of chloride in serum water, corrected for the Donnan equilibrium. The sum of the 'fibre' and 'non-fibre' water is the total extracellular water and this has been subtracted from the total amount of water in the tissue to give the amount of intracellular water. The basis of the calculation of the volume of 'non-fibre' water is the assumption that all the chloride in the tissue is outside the cells. Although this assumption may not be valid in some species at certain ages (Eichelberger *et al.* 1943), it nevertheless yields a picture of the changes in the distribution of skin water during development which can be correlated with the changes in histological structure that take place during this process.

Table 12 shows the amount of 'fibre', 'non-fibre' and intracellular water in the skin of man and the pig at the different ages expressed as a percentage of the amount of total water. At all ages, in both species, over 66% of the total water in the organ was accounted for by extracellular fluid. The distribution of this extracellular fluid changed with the progress of development, for the proportion of it associated with the collagen fibres increased along with the increase in collagen, whereas the 'non-fibre' extracellular water decreased. In human skin the percentage of the total water in the cells increased to a maximum at the time of birth, and decreased to a lower level in the adult. This change roughly paralleled the change in the concentration of cellular substances in this species (Table 1). In the pig the proportion of cell water was very small in the youngest foetus and in the adult, and here again the concentrations of the cellular constituents were lower at these ages than at the intermediate ones (Table 2).

The fall in the proportion of non-collagen nitrogen during growth is due to a fall in the contribution of the proteins of the cells and ground substance to the total nitrogen in the tissue. The number of cells visible in a section of human foetal skin makes it seem likely that cell proteins contribute a fairly large proportion of the non-

Table 12. *Distribution of skin water*

The 'fibre' and 'non-fibre' water values were calculated from the average values for collagen and Cl per kg. of skin given in Table 1, and the results are expressed as a percentage of the total water. 'Fibre' water + 'non-fibre' water = total extracellular water. Total water − total extracellular water = cell water.

	Extracellular water			
	'Fibre' water	'Non-fibre' water	Total	Cell water
Human skin				
Foetus 20–22 weeks	2·5	90·6	93·1	6·9
Baby (New-born	19·4	47·3	66·7	33·3
Baby (3–6 months	55·7	34·0	89·7	10·3
Adult	63·7	23·1	86·8	13·2
Pig skin				
Foetus (46 days	0·4	98·0	98·4	1·6
Foetus (90 days	3·9	84·0	87·9	12·1
Piglet (New-born	11·0	78·0	89·0	11·0
Piglet (3 weeks	23·2	56·5	79·7	20·3
Piglet (4–6 weeks	38·0	43·0	81·0	19·0
Adult	68·0	23·4	91·4	8·6

collagen nitrogen before birth. The space between the cells must be filled with extracellular material of the same general composition as that in the other organs and tissues of the body. The amount of collagen in the skin at this age is small when compared with that in the adult tissue and, in fact, sections stained for collagen by the Van Gieson method did not show the presence of any mature collagen fibres. Sections stained with a silver-impregnation technique showed that the collagen estimated in human foetal skin was in the form of reticulin fibres, which formed a fine interlacing network in which the cells of the corium were embedded. Since the amount of collagen in foetal skin was small, there was a correspondingly small amount of extracellular water associated with it; most of the extracellular water was therefore associated with the mucopolysaccharides and non-fibrous proteins of the extracellular phase. This gave the skin an 'oedematous' appearance when it was removed from the body, and the sections show that the skin was thicker in the human foetus than in the full-term baby.

The skin of the new-born baby contains fewer cells, loosely scattered in the corium, but glands of mature appearance are present and the epidermis is also more mature than in the foetus. The glands and epidermis together probably contribute to the increase in the amount of potassium during the latter part of gestation. At birth the corium is loosely packed with collagen fibres.

The thickness of the skin increases during post-natal growth, and the combination of this with a decrease in cell density in the corium is largely responsible for the fall in the level of potassium in the skin after a post-natal age of 7 months.

These descriptions have been mostly concerned with human skin but the histological changes in pig skin during growth are, on the whole, very similar. There is much less collagen in the skin of the pig than in that of the baby, at birth.

Much of the interest in the composition of normal skin has been in its function as a depot for mobile water and salt (see Rothman, 1954). The whole of the extracellular water in the skin has been described as 'available water' (Flemister, 1941–42), and it has been shown that the amount of water in the skin changes more than that of skeletal muscle, for example, during dehydration (Rothman, 1954). It does not appear to have been shown whether this water represents a decrease in both fibre and non-fibre extracellular water, or if it is, in fact, drawn only from the latter. It would seem to be unlikely that the fibre water would be 'available' since analyses of tendon have shown that collagen is always associated with a constant amount of water and electrolytes (Manery *et al.* 1938).

After the skeletal muscles the skin is the largest soft tissue of the body, and the skin, the skeleton and the skeletal muscles in the adult human body together contribute about 70% of the body weight (Forbes & Lewis, 1956). Table 13 shows the proportion of the total nitrogen in the body contributed by each of these tissues and also the proportion of the total nitrogen in the body contributed by the collagen in them. The values for the weights of fat-free skin, skeleton and skeletal muscle and of the whole fat-free body have been taken from the data of Forbes, Cooper & Mitchell (1953), Forbes & Lewis (1956) and Forbes, Mitchell & Cooper (1956). The values for total nitrogen have been calculated from the data published by these workers for the same subjects. The amounts of collagen nitrogen in the whole skin and skeletal muscles have been calculated from the percentage of the total nitrogen contributed by collagen in these tissues as found in the present study and in that of Dickerson & Widdowson (1960). The value for the skeleton was obtained from the percentage of the total nitrogen accounted for by collagen in the adult human femur (J. W. T. Dickerson, unpublished data), on the assumption that this bone is representative of the whole skeleton. The results show that the three major tissues of the body contribute over 70% of the total nitrogen in the body and that the collagen of the skin and skeleton account for 25% of the total nitrogen. Since considerable amounts of collagen are also present in the gastrointestinal tract and in the lungs, it seems reasonable to suppose that at least a quarter of the protein in the adult human body is outside the cells (Neuberger, 1955). An increase in the amount of extracellular protein in the skin may well have accounted for some of the stored nitrogen in subjects who were recovering from a protein deficiency (Holmes, Jones & Stanier, 1954).

Table 13. *Contribution of the skin, skeleton and skeletal muscle to the total nitrogen in the adult human body*

	Wt. (fat-free) (kg.)	Total N (g.)	Collagen N (g.)	N in organ (% of total N in body)	Collagen N in organ (% of total N in body)
Total body	48·1	1840	—	—	—
Skin	3·3	176	158	9·5	8·5
Skeleton	8·0	331	271	18·1	14·8
Skeletal muscle	23·1	856	28	46·3	1·5

The skin probably accounts for a greater proportion of the body weight in the human foetus and new-born baby than it does in the adult (Wilmer, 1940), and the changes in its composition, particularly during early post-natal life, emphasize the importance of the contribution of this organ to the changes in the composition, of the whole body during development. McCance & Widdowson (1957) have stressed the importance of growth in the homoeostasis of the new-born. The skin may be as important as skeletal muscle in this regard for, in man, over 30 g. of nitrogen was found to be deposited in 1 kg. of fat-free skin during the first 3–5 months after birth, and in the pig 12 g. of nitrogen was deposited in each kg. of fat-free skin during the first 3 weeks of post-natal growth. This deposition of nitrogen not only relieves the kidney of dealing with it, but it also equips the skin better to carry out its own responsibilities of protecting the underlying tissues from desiccation, heat loss and the entry of noxious organisms.

SUMMARY

1. Skin, cardiac muscle, liver, kidney and brain of man and the pig have been analysed in the foetus, in the new-born, during the suckling period and in the adult.

2. Development was associated with a fall in the proportion of water and an increase in the proportion of nitrogen in all tissues; foetal heart muscle, and foetal liver and kidneys reached their adult composition before skin and skeletal muscle, and it is suggested that this is related to their early functional development.

3. Next to skeletal muscle the skin is the largest soft tissue of the body, and changes in its composition make a significant difference to the composition of the body as a whole. The proportion of collagen per kilogram of skin increased during development, both in absolute terms and as a percentage of the total nitrogen. The cellular constituents were at a maximum at about the time of birth.

4. The hearts of the still-born babies contained considerably less potassium than did the foetal hearts. Two possible explanations are put forward for this anomalous finding.

5. Human brain contained more water and less nitrogen and phosphorus than pig brain at corresponding stages of development. This may be due to a larger amount of grey matter in human-brain substance.

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REFERENCES

Alexander, L. C., Boyle, A. J., Iseri, L. T., McCaughey, R. S. & Myers, G. B. (1950). *J. Lab. clin. Med.* **36**, 796.
Amberson, W. R., Nash, T. P., Mulder, A. G. & Binns, D. (1938). *Amer. J. Physiol.* **122**, 224.
Bezold, A. von (1857). *Z. wiss. Zool.* **8**, 487.
Clarke, N. E. & Mosher, R. E. (1952). *Circulation*, **5**, 907.
Cullen, G. E., Wilkins, W. E. & Harrison, T. R. (1933). *J. biol. Chem.* **102**, 415.
Davies, F., Davies, R. E., Francis, E. T. B. & Whittam, R. (1952). *J. Physiol.* **118**, 276.
Davson, H. & Spaziani, E. (1959). *J. Physiol.* **149**, 135.
Dickerson, J. W. T. & Widdowson, E. M. (1960). *Biochem. J.* **74**, 247.
Economou-Mavrou, C. & McCance, R. A. (1958). *Biochem. J.* **68**, 573.
Eichelberger, L., Eisele, C. W. & Wertzler, D. (1943). *J. biol. Chem.* **151**, 177.
Eisele, C. W. & Eichelberger, L. (1945). *Proc. Soc. exp. Biol., N.Y.*, **58**, 97.
Flemister, L. J. (1941–42). *Amer. J. Physiol.* **135**, 430.
Flexner, J. B. & Flexner, L. B. (1950). *Anat. Rec.* **106**, 413.
Flexner, L. B. & Flexner, J. B. (1949). *J. cell. comp. Physiol.* **34**, 115.
Forbes, G. B. & Lewis, A. M. (1956). *J. clin. Invest.* **35**, 596.
Forbes, R. M., Cooper, A. R. & Mitchell, H. H. (1953). *J. biol. Chem.* **203**, 359.
Forbes, R. M., Mitchell, H. H. & Cooper, R. A. (1956). *J. biol. Chem.* **223**, 969.
Hamilton, W. J., Boyd, J. D. & Mossman, H. W. (1945). *Human Embryology*. Cambridge: W. Heffer and Sons Ltd.
Hercus, V. M., McDowall, R. J. S. & Mendel, D. (1955). *J. Physiol.* **129**, 177.
Holmes, E. G., Jones, E. R. & Stanier, M. W. (1954). *Brit. J. Nutr.* **8**, 173.
Kennedy, G. C. & Pearce, W. (1958). *J. Endocrin.* **17**, 149.
Kerpel-Fronius, E. (1937). *Z. Kinderheilk.* **58**, 726.
Lowry, O. H., Gilligan, D. R. & Katersky, E. M. (1941). *J. biol. Chem.* **139**, 795.
McCance, R. A. & Widdowson, E. M. (1956*a*). *Clin. Sci.* **15**, 409.
McCance, R. A. & Widdowson, E. M. (1956*b*). *Quart. J. exp. Physiol.* **41**, 1.
McCance, R. A. & Widdowson, E .M. (1957). *Lancet*, ii, 585.
McCance, R. A. & Widdowson, E. M. (1959). *J. Physiol.* **147**, 124.
McDowall, R. J. S., Munro, A. F. & Zayat, A. F. (1955). *J. Physiol.* **130**, 615.
Manery, J. F., Danielson, I. S. & Hastings, A. B. (1938). *J. biol. Chem.* **124**, 359.
Manery, J. F. & Hastings, A. B. (1939). *J. biol. Chem.* **127**, 657.
Mangun, G. H., Reichle, H. S. & Myers, V. C. (1941). *Arch. intern. Med.* **67**, 320.
Morrison, A. B. (1959). *J. clin. Invest.* **38**, 1769.
Moulton, C. R. (1923). *J. biol. Chem.* **57**, 79.
Neuberger, A. (1955). *Symp. Soc. exp. Biol.* **9**, 72.

Oster, R. H. & Amberson, W. R. (1939). *J. biol. Chem.* **131**, 19.
Rossiter, R. J. (1955). In *Neurochemistry*, p. 11. Ed. by Elliott, K. A. C., Page, I. H. & Quastel, J. H. Springfield, Ill.: C. C. Thomas.
Rothman, S. (1954). *Physiology and Biochemistry of the Skin*, p. 493. Chicago, Ill.: University of Chicago Press.
Spray, C. M. & Widdowson, E. M. (1951). *Brit. J. Nutr.* **4**, 332.
Stanier, M. W. (1960). *J. Physiol.* **151**, 472.
Stewart-Wallace, A. M. (1939). *Brain*, **62**, 426.
Stubbs, J. & Widdas, W. F. (1959). *J. Physiol.* **148**, 363.
Usher, R. (1959). *Pediatrics, Springfield*, **24**, 562.
Westin, B., Nyberg, R. & Enhörning, G. (1958). *Acta Paediat., Stockh.*, **47**, 339.
Widdowson, E. M. & McCance, R. A. (1956). *Clin. Sci.* **15**, 1361.
Widdowson, E. M., McCance, R. A. & Spray, C. M. (1951). *Clin. Sci.* **10**, 113.
Widdowson, E. M. & Spray, C. M. (1951). *Arch. Dis. Childh.* **26**, 205.
Wilkins, W. E. & Cullen, C. E. (1933). *J. clin. Invest.* **12**, 1063.
Wilmer, H. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 545.
Yannet, H. & Darrow. D. C. (1940). *J. biol. Chem.* **134**, 721.

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Insulin and an Inhibitor of Glucose Uptake, in Protein Fractions of Normal Human Plasma

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Experiments in which the proteins of normal human serum or ox serum were fractionated by zone electrophoresis on columns of treated cellulose have suggested that insulin in serum migrates with fractions containing α_1-, β- and γ-globulins (Randle & Taylor, 1958; Taylor & Randle, 1959). Further subfractionation of the β- and γ-globulins suggested that insulin was associated predominantly with β- rather than γ-globulins and it seemed possible that insulin in γ-globulins is derived from contamination with β-globulins as a result of zone spreading during electrophoresis. In addition, evidence was obtained for the presence of an inhibitor of glucose uptake by isolated rat diaphragm *in vitro*, migrating in the α_2-globulin fraction. Detection of this inhibitor was made difficult by contamination of the α_2-globulin fraction with insulin derived possibly from adjacent α_1- and β-globulins (Randle & Taylor, 1958).

We have now sought to confirm these conclusions by an alternative method of protein fractionation, with ethanol at low temperatures, which effects a much better separation of β- and γ-globulin fractions of normal human serum than zone electrophoresis. In order to demonstrate the presence of the inhibitor with certainty it was necessary to subfractionate one of the fractions prepared with ethanol, by an improved method of zone electrophoresis. Details of this method of zone electrophoresis are given in the Appendix.

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EXPERIMENTAL

Blood samples. Human blood was drawn from an antecubital vein of non-fasting subjects and diluted immediately with 0·173 vol. of acid–citrate–dextrose (Lever *et al.* 1951). The diluted plasma was then separated by centrifuging.

Fractionation of plasma proteins. The proteins of diluted plasma were first separated into three fractions with ethanol at $-5°$ by the method of Lever *et al.* (1951). The fractions obtained have been designated according to Lever *et al.* as fraction I + III (mainly α_2- and β-globulins and fibrinogen), fraction II (γ-globulins) and fraction IV, V and VI (mainly albumin and α-globulins with a little β-globulin). The fractionation was confirmed in most instances by paper electrophoresis of the fractions, employing the vertical-tank method of Flynn & de Mayo (1951) and staining the protein with bromophenol blue by the method of Henry, Golub & Sobel (1957). The protein fractions were dialysed for 30 hr. against distilled water, in Visking tubing, at room temperature and freeze-dried.

Fraction IV, V and VI was subfractionated by zone electrophoresis at pH 8·4, on columns of treated cellulose, 50 cm. long and 2·0 cm. in diameter, (Porath, 1956) with the phosphate–borate buffer of Campbell & Stone (1956). The faster-moving proteins (albumin and α_1-globulin) were allowed to move off the column during

electrophoresis into a glass cell attached to the bottom of the column, from which they were swept by a slow stream of buffer into an automatic fraction collector. The design of this cell and its mode of operation are described in a note at the end of this paper. Electrophoresis for about 30 hr. at 750 v and 40 ma sufficed to displace the faster-moving proteins from the column; electrophoresis was then discontinued and the remaining protein was displaced from the column with phosphate–borate buffer. The protein content of each fraction was assayed by measurement of E at 280 mμ in the Beckman spectrophotometer. The individual fractions were then pooled to yield three main fractions (Fig. 1): albumin + α_1-globulin, α_2-globulin and β-globulin, and these were dialysed against distilled water and freeze-dried.

For assay of biological activity, the freeze-dried protein samples were dissolved in bicarbonate-buffered medium (Gey & Gey, 1936) containing glucose (2·5 mg./ml.). The volume of medium was three times the volume of plasma from which the fractions were prepared. Fraction I + III (containing fibrinogen) clotted at this stage; the clot was allowed to form and it was then removed by centrifuging.

Diaphragm. Diaphragm muscle was obtained from male albino Wistar rats, wt. 100–150 g., which had been fasted for 18–24 hr. The procedure for preparation and incubation of diaphragms was that described by Randle (1954).

Assay of biological activity. The uptake of glucose or synthesis of glycogen by hemidiaphragms incubated for 90 min. at 37° in buffer containing a protein fraction (one hemidiaphragm in 1 ml. of medium) was compared with that of hemidiaphragms incubated in buffer alone. Glucose uptake was calculated as milligrams of glucose disappearing from the medium/g. of wet diaphragm/hr. of incubation. Insulin was deemed to be present if a fraction stimulated glucose uptake or glycogen synthesis significantly (cf. Taylor & Randle, 1959). The term inhibitor is used to describe the factor or factors present in those fractions which depressed glucose uptake significantly.

Glucose. This was estimated in samples (0·2 ml.) of incubation medium by Somogyi's (1945) modification of Nelson's method.

Glycogen. This was isolated in crude form from diaphragm muscle by the method of Walaas & Walaas (1950) and assayed photometrically by reaction with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

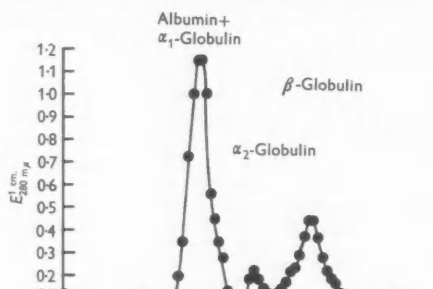

Fig. 1. Zone electrophoresis of fraction IV, V and VI.

RESULTS

The effects of protein fractions of normal human plasma on the uptake of glucose by diaphragm are shown in Tables 1 and 3. Fraction I + III (principally α_2- and β-globulins) consistently stimulated uptake of glucose by diaphragm; this fraction also enhanced synthesis of glycogen by diaphragm. Fraction II (γ-globulins) had no significant effect on glucose uptake. The effect of fraction IV, V and VI (mainly albumin and α-globulins with some β-globulins) was variable and whereas some samples depressed uptake of glucose, other samples either stimulated glucose uptake or were without effect. The reason for this variable effect of fraction IV, V and VI became apparent when subfractions were prepared by zone electrophoresis and tested for biological activity (Fig. 1 and Table 2). The subfraction containing slower-moving albumin and α_1-globulins stimulated uptake of glucose, whereas subfractions containing α_2- or β-globulins depressed uptake of glucose. Further, the sum of the effects of the individual subfractions was very similar to the effect of the fraction from which they were prepared (Table 2). The identity of the subfractions was confirmed by paper electrophoresis.

We conclude from these results that fraction I + III contains insulin and that fraction IV, V and VI contains both insulin (present in the albumin–α_1-globulin subfraction) and an inhibitor of glucose uptake (present in the α_2- and β-globulin subfractions).

Table 1. *Effect of protein fractions of normal human plasma on uptake of glucose by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951). For definition of fractions see Experimental section. For differences marked thus * $P < 0{\cdot}001$; † $P < 0{\cdot}01 > 0{\cdot}001$; ‡ $P < 0{\cdot}05 > 0{\cdot}01$. For other differences $P > 0{\cdot}05$. Each difference is derived from the mean of six observations.

	Glucose uptake as difference (mg. of glucose/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference ± S.E. of difference		
Sample	Fraction I + III	Fraction II	Fraction IV, V and VI
1	1·5 ± 0·29*	—	0·8 ± 0·21†
2	1·2 ± 0·74	0·5 ± 0·33	−2·1 ± 0·40*
3	0·1 ± 0·20	0·4 ± 0·40	−0·6 ± 0·24‡
4	1·4 ± 0·24*	0·1 ± 0·30	−0·6 ± 0·38
5	1·8 ± 0·51†	0·1 ± 0·51	−0·4 ± 0·55
6	1·7 ± 0·45†	0·6 ± 0·41	−1·1 ± 0·39‡
Mean	1·3 ± 0·25†	0·3 ± 0·3	−0·7 ± 0·39

Table 2. *Effect of subfractions of fraction IV, V and VI from normal human plasma on the uptake of glucose by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951) and subfractionated by zone electrophoresis on columns of treated cellulose (see Experimental section). For definition of fractions see Experimental section and Fig. 1. For differences marked thus * $P < 0.001$; † $P < 0.01 > 0.001$. For other differences $P > 0.05$. Each difference is derived from means of six observations.

Sample	Subfraction	Glucose uptake as difference (mg. of glucose/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference ± S.E. of difference
1	—	−0·5±0·29
	Albumin + α_1-globulin	0·1±0·62
	α_2-Globulin	−0·6±0·70
	β-Globulin	0·1±0·62
2	—	−0·4±0·75
	Albumin + α_1-globulin	1·9±0·48†
	α_2-Globulin	−1·5±0·39†
	β-Globulin	−1·2±0·35†
3	—	−0·3±0·31
	Albumin + α_1-globulin	1·5±0·32*
	α_2-Globulin	−0·6±0·39
	β-Globulin	−1·1±0·34†
Pooled	—	−0·4±0·31
	Albumin + α_1-globulin	1·2±0·27*
	α_2-Globulin	−0·9±0·28†
	β-Globulin	−0·8±0·25†

Table 3. *Effect of protein fraction I+III, from normal human plasma, on the deposition of glycogen by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951). For definition of fractions see Experimental section. For differences marked * $P < 0.001$; † $P < 0.01 > 0.001$; ‡ $P < 0.02 > 0.01$. For other differences $P > 0.05$. Each difference is derived from means of six observations.

Sample	Glycogen deposition as difference (mg. of glycogen/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference ± S.E. of difference
1	0·9±0·3‡
2	0·5±0·34
3	3·0±0·71†
Pooled	1·5±0·27*

DISCUSSION

Earlier studies, in which protein fractions were prepared from normal human serum by column electrophoresis, had shown that insulin is predominantly associated with α_1- and β-globulins but that some insulin was also present in γ-globulins (Randle & Taylor, 1958). In these studies, insulin in the γ-globulin fraction could have been derived from contamination with β-globulins as a result of spreading of zones during elution. In the present study a more effective separation of serum-protein fractions has been attained by a combination of fractional precipitation and an improved method of column electrophoresis. Under these conditions insulin could not be detected in γ-globulins and the results confirm that in normal human serum insulin is present in fractions containing α_1-globulins and β-globulins (cf. Beigelman *et al.* 1956; Bolinger, van der Geld & Willebrands, 1959; Beigelman, 1958).

A substance which inhibits uptake of glucose by diaphragm has been identified in α_2- and β-globulin fractions prepared from normal human serum by a combination of fractional precipitation and zone electrophoresis. In earlier studies with zone electrophoresis alone, evidence was obtained for the presence of such an inhibitor of glucose uptake in α_2-globulin fractions, but detection of the inhibitor was difficult, possibly because of contamination with insulin from adjacent α_1- and β-globulins. In the present study much of the insulin was separated from the inhibitor by preliminary fractional precipitation and the inhibitor was then readily separated from the remaining insulin by zone electrophoresis. Interest in this inhibitor lies in its possible relation to growth hormone, for, whereas treatment of the rat with growth hormone leads to diminished uptake of glucose by diaphragm, the hormone does not inhibit glucose uptake when added *in vitro* (Park, Brown, Cornblath, Daughaday & Krahl, 1952). Preliminary

studies have suggested that the inhibitor is not present in α_2- or β-globulin fractions prepared from the plasma of hypophysectomized patients but that it is present after treatment with human growth hormone. This inhibitor appears to be different from the insulin antagonist identified by Vallance-Owen, Dennes & Campbell (1958) which was associated with albumin fractions of human serum and which did not depress uptake of glucose in the absence of added insulin. Baird & Bornstein (1958) have noted inhibition of glucose uptake by diaphragm with residues from human plasma after extraction with acid–ethanol. We do not know whether our inhibitor is similar to that described by Baird & Bornstein.

The present studies again emphasize that assays of insulin in plasma based on the effects of unfractionated plasma on glucose uptake of diaphragm are of little value since such samples are likely to contain both insulin and inhibitors of glucose uptake (cf. Randle, 1954).

SUMMARY

1. Protein fractions have been prepared from normal human plasma by fractional precipitation with ethanol and an improved method of zone electrophoresis on columns of treated cellulose, and their effect on the uptake of glucose by isolated rat diaphragm has been investigated.

2. Insulin was detected in a protein fraction containing slower-moving albumin and α_1-globulins and in one containing α_2- and β-globulins. No insulin could be detected in γ-globulins.

3. An inhibitor of glucose uptake by isolated diaphragm was identified in a fraction containing α_2- and β-globulins.

We wish to thank Professor F. G. Young for his continued interest and encouragement. Mrs W. Thorne is thanked for skilled technical assistance. The cost of these investigations was defrayed, in part, by grants for research expenses from the Medical Research Council to (P.J.R.) and from the British Diabetic Association (to K.W.T.).

APPENDIX

A Method of Continuous Elution during Column Electrophoresis

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Porath (1956) has pointed out that zone spreading on elution is a serious disadvantage when protein mixtures are fractionated by column electrophoresis. Porath, Lindner & Jerstedt (1958) have suggested that electrophoresis be carried out in such a way that the more rapidly moving components pass from the column into a cell, from which they are swept by a stream of buffer into an automatic fraction collector. A similar method, which has been in use for some time in this Laboratory, is described in this note.

The general design of the cell is shown in Fig. 1. The cell consists of a short tube (length, 5 cm.; internal diameter, 2 cm.) bearing at its upper end a B24 standard ground-glass joint and closed at its lower end by a single layer of Visking dialysis tube stretched across a glass lip and held in place by a rubber band. Two glass side arms are fitted to the cell, the lower of which allows buffer to flow into the cell, the upper tube serving to conduct the eluate to the fraction collector. A baffle plate, consisting of a single layer of Visking membrane perforated with pin holes, is fixed in position between the side arms by means of a split polythene ring.

The whole cell (and its appendages) is fitted on the lower end of a conventional glass electrophoresis column (length, 50 cm.; internal diameter, 2 cm.) and immersed in a tank of buffer containing the anode.

During electrophoresis a slow stream of buffer is passed through the cell at a rate sufficient to remove the faster-moving components as they pass into the cell during electrophoresis [in the paper (above) by Vargas, Taylor & Randle the rate was

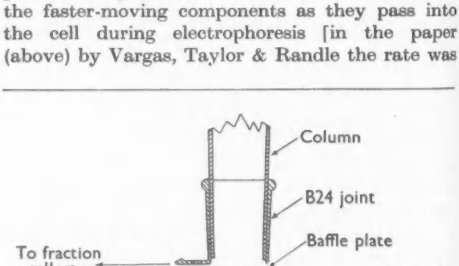

Fig. 1. Design of cell.

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approx. 16 ml./hr. during removal of the albumin]. As electrophoresis proceeds, with serum, it is necessary to slow the stream of buffer once the albumin has been removed, in order to avoid undue dilution of more slowly migrating components.

When this method was applied to electrophoresis of normal human serum, the resolution of faster-moving components was greatly improved, so that, for example, a distinct pre-albumin peak was obtained which was clearly demarcated from the albumin peak in almost all instances.

REFERENCES

Baird, C. W. & Bornstein, J. (1958). *Lancet*, i, 1111.
Beigelman, P. M. (1958). *Diabetes*, **7**, 365.
Beigelman, P. M., Antoniades, H. N., Goetz, F. C., Renold, A. E., Oncley, J. L. & Thorn, G. W. (1956). *Metabolism*, **5**, 35.
Bolinger, P. E., van der Geld, G. & Willebrands, A. F. (1959). *Metabolism*, **8**, 39.
Campbell, P. N. & Stone, N. E. (1956). *Biochem. J.* **62**, 9P.
Flynn, F. V. & de Mayo, P. (1951). *Lancet*, ii, 235.
Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Henry, R. J., Golub, O. J. & Sobel, C. (1957). *Clin. Chem.* **3**, 49.
Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K. & Schultz, E. L. (1951). *J. clin. Invest.* **30**, 99.
Park, C. R., Brown, D. H., Cornblath, M., Daughaday, W. H. & Krahl, M. E. (1952). *J. biol. Chem.* **199**, 151.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Porath, J., Lindner, E. B. & Jerstedt, S. (1958). *Nature, Lond.*, **182**, 744.
Randle, P. J. (1954). *Brit. med. J.* **1**, 1237.
Randle, P. J. & Taylor, K. W. (1958). *J. Endocrin.* **17**, 387.
Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Taylor, K. W. & Randle, P. J. (1959). *J. Endocrin.* **19**, 221.
Vallance-Owen, J., Dennes, E. & Campbell, P. N. (1958). *Lancet*, ii, 336.
Walaas, O. & Walaas, E. (1950). *J. biol. Chem.* **187**, 769.

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Studies on Vitamin E

5. LIPID PEROXIDATION IN DIALURIC ACID-INDUCED HAEMOLYSIS OF VITAMIN E-DEFICIENT ERYTHROCYTES*

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(*Received* 9 *March* 1960)

Dialuric acid-induced haemolysis of erythrocytes from vitamin E-deficient rats was discovered by Rose & György (1950, 1952), who postulated that the haemolytic agent was an intermediate in the reversible oxidation-reduction system of dialuric acid and alloxan but was not hydrogen peroxide itself.

Red pigments are produced when 2-thiobarbituric acid reacts with oxidized lipids (Bernheim, Bernheim & Wilbur, 1947) or, more probably, with their aldehydic breakdown products (Glavind & Hartmann, 1951), and the formation of both red and yellow pigments from non-lipids has been discussed by Patton & Kurtz (1951) and by Landucci, Pouradier & Duranté (1955). The thiobarbituric acid reaction was used by Horwitt, Harvey, Duncan & Wilson (1956) in a study of hydrogen peroxide-induced haemolysis of erythrocytes from humans on a diet low in vitamin E, and by Tappel & Zalkin (1959, 1960) to demonstrate the presence of lipid peroxides in mitochondria and microsomes from vitamin E-deficient rats. Carpenter, Kitabchi, McCay & Caputto (1959) also used it to show that peroxide formation in liver homogenates from vitamin E-deficient rats was paralleled by decreased synthesis of ascorbic acid.

The protective effects of tocopherols and metal ions against dialuric acid-induced haemolysis have been discussed by Bunyan, Green, Edwin & Diplock (1960). This paper deals with the results of fuller investigations into the haemolysis of vitamin E-deficient erythrocytes and describes the use of the 2-thiobarbituric acid reaction to study the relation between lipid peroxidation and the haemolytic phenomenon.

* Part 4: *Biochem. J.* **76**, 563.

EXPERIMENTAL

Animals. Deficient animals were 3- to 5-month-old male rats of the Norwegian hooded strain which had received the vitamin E-free diet since they were 13 days old. Control rats were males from the stock colony.

Vitamin E-free diet, buffer pH 7·4 *and dialuric acid solution.* These were as described by Bunyan *et al.* (1960);

the buffer consisted of 25 ml. of 0·2 M-KH_2PO_4 solution and 19·7 ml. of 0·2 M-NaOH solution diluted to 100 ml. with water.

2-Thiobarbituric acid solution. 2-Thiobarbituric acid (0·67 g.) was dissolved in 100 ml. of warm water and the solution was cooled and mixed with 100 ml. of acetic acid.

α-Tocopherol suspension. A weighed amount of α-tocopherol was mixed with four times as much Tween 80 and then shaken with 0·9% NaCl solution.

Other additives. Metal ions and 2:6-di*tert.*-butyl-4-methylphenol were used as solutions of the appropriate material in 0·9% NaCl. L-Thyroxine (sodium salt) was dissolved in 0·01 N-NaOH and diluted with 0·9% NaCl. Crude ox-liver catalase was prepared by the method of Tauber & Petit (1952) and it was found that 1 g. of the preparation decomposed 200 g. of 100% H_2O_2 in 10 min. at 25° in an inert atmosphere. A portion (0·2 ml.) of catalase suspension (2·5 mg. in 1 ml. of buffer, pH 7·4) was added to 0·8 ml. of 25% erythrocyte suspension just before adding dialuric acid.

Carbon monoxide. This was produced by treating formic acid with conc. H_2SO_4 at 70° and was purified by passage through a tower of KOH pellets. Erythrocytes were treated by filling the air space in the stoppered tube with CO several times, with inversion of the tube each time. In some experiments, the resulting CO atmosphere was removed by blowing in air or by bubbling nitrogen through the cell suspension. The dialuric acid reaction was then applied at once.

Erythrocyte suspension. Blood was collected from the rat's tail into NaCl–citrate solution (0·9 g. of NaCl and 0·6 g. of trisodium citrate/100 ml.) and centrifuged at 2500 rev./min. The cells were washed twice with 0·9% NaCl and finally made up to a 20% suspension in 0·9% NaCl.

Pretreatment of the erythrocyte suspension. The various test substances were incubated at 37° for 1 hr. with 1 ml. of the 20% suspension (Table 2). The mixture was then centrifuged at 2500 rev./min., the supernatant was removed and the volume was made up to 1 ml. again with 0·9% NaCl.

Dialuric acid-induced haemolysis followed by the 2-thiobarbituric acid reaction. The 20% erythrocyte suspension (1 ml.) was incubated, after pretreatment if necessary, for 15 min. at 37° with 1 ml. of dialuric acid solution (3·3 mM unless otherwise stated). The mixture was centrifuged at 2500 rev./min. A portion (0·1 ml.) of the supernatant was removed and diluted with 7 ml. of water, and the extinction of the solution was measured against a water blank (EEL absorptiometer; Ilford filter, 605; $\lambda_{max.}$ 550 mμ). Calibration for 100% haemolysis was carried out by haemolysing 0·1 ml. of the original 20% suspension with 14 ml. of water. The remaining supernatant and sediment were shaken with 1 ml. of 10% trichloroacetic acid solution to precipitate proteins and the mixture was then filtered. The filtrate (2 ml.) was treated with 2·5 ml. of 2-thiobarbituric acid solution for 5 min. at 100° followed by 5 min. at room temperature (20–25°). The production of a pink colour ($\lambda_{max.}$ 535 mμ) was taken to indicate lipid peroxidation and the extinction was measured with Ilford filter 605. A yellow colour ($\lambda_{max.}$ 450 mμ), which can result from the action of thiobarbituric acid on dialuric acid in the absence of erythrocytes, was regarded as evidence of a negative reaction and was not measured.

Preparation of erythrocyte stroma and stroma-free haemolysates. Blood was collected as described above and the erythrocytes were washed three times with ice-cold 0·9% NaCl by centrifuging at 3000 rev./min. Haemolysis was effected by mixing with 10 vol. of ice-cold water. The haemolysate was allowed to stand for 30 min. and then centrifuged either for 5 min. at about 1000 *g* (3000 rev./min.) or for 15 min. at about 20 000 *g* (MSE Superspeed 25 centrifuge). About three-quarters of the clear supernatant was removed for testing. The stroma fraction was washed repeatedly with cold 0·9% NaCl or water by centrifuging at 3000 rev./min. until no more haemoglobin dissolved; some remained adherent to the stroma. The stromal material was then resuspended in 0·9% NaCl to a concentration equivalent to that of the original erythrocyte suspension. Haemolysis and peroxide formation varied slightly from day to day but most results were derived by direct comparison with a given test.

Reaction of erythrocyte fractions with dialuric acid and thiobarbituric acid. Stroma suspensions in 0·9% NaCl or stroma-free haemolysate were treated with dialuric acid solution followed by thiobarbituric acid as described for intact erythrocytes.

Spectral examinations. The haemoglobin spectrum during haemolysis was observed by means of a direct-vision spectroscope (R. and J. Beck Ltd.) and quantitative measurements were made on the Hilger Uvispek spectrophotometer. Reduced haemoglobin and methaemoglobin, for purposes of comparison, were produced by treating haemolysed vitamin E-deficient erythrocytes with traces of $Na_2S_2O_4$ and $K_3Fe(CN)_6$ respectively.

Chemicals. Dialuric acid and L-thyroxine (sodium salt) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.; 2-thiobarbituric acid from British Drug Houses Ltd.; α-tocopherol from Roche Products Ltd. and 2:6-di*tert.*-butyl-4-methylphenol from William Pearson Ltd., Hull.

RESULTS

Intact erythrocytes

The 5% erythrocyte suspension used in our previous study (Bunyan *et al.* 1960) was here increased to 20% in order to enhance the thiobarbituric acid reaction. In preliminary tests a higher concentration of dialuric acid was also used but this did not induce haemolysis. An investigation of various concentrations of dialuric acid showed that solutions between 1·6 and 3·3 mM were most effective and that haemolysis was paralleled by the formation of lipid peroxides. Peroxides were not found in erythrocytes haemolysed by dilution in water (Table 1).

Substances previously found to be protective against dialuric acid-induced haemolysis (α-tocopherol, Co^{2+} ions, Mn^{2+} ions, $Cr_2O_7^{2-}$ ions, CrO_4^{2-} ions and 2:6-di*tert.*-butyl-4-methylphenol) also prevented lipid peroxidation, the protection due to α-tocopherol and $Cr_2O_7^{2-}$ ions persisting even after three washes with 0·9% sodium chloride. The antagonism of SeO_3^{2-} ions towards tocopherol protection was also shown (Table 2). L-Thyroxine, investigated here for the first time, gave full

Table 1. *Haemolysis and lipid peroxidation with varying concentrations of dialuric acid solution and erythrocyte suspension*

For details see text.

Dialuric acid soln. (mM)	Vitamin E-deficient erythrocyte suspension (%)	Haemolysis (%)	Thiobarbituric acid reaction (E at 550 mμ)
13·2	20	53	10
6·6	20	92	12
3·3	20	94	17
2·5	20	93	22
1·6	20	95	19
Nil (haemolysed with water)	20	100	—*
13·2	10	0	3
13·2	4	0	—*

* Yellow.

Table 2. *Effect of various substances on haemolysis and lipid peroxidation*

For details see text.

Pretreatment	Haemolysis (%)	Thiobarbituric acid reaction (E at 550 mμ)
Nil (normal erythrocytes)	0	—*
Vitamin E-deficient erythrocytes		
Nil (and no dialuric acid)	0	—*
Nil	83	14
α-Tocopherol (4 μg.)	0	—*
α-Tocopherol (4 μg.) followed by three washes with 0·9% NaCl	0	—*
α-Tocopherol (2 μg.)	8	—*
SeO_3^{2-} (as Na_2SeO_3) (64 μg.)	67	15
SeO_3^{2-} (64 μg.) + α-tocopherol (2 μg.)	73	15
L-Thyroxine (sodium salt) (100 μg.)	2	—*
Co^{2+} (as $CoCl_2$) (200 μg.)	0	—*
Mn^{2+} (as $MnSO_4$) (200 μg.)	0	—*
$Cr_2O_7^{2-}$ (as $K_2Cr_2O_7$) (300 μg.)	0	—*
$Cr_2O_7^{2-}$ (300 μg.) followed by three washes with 0·9% NaCl	0	—*
CrO_4^{2-} (as K_2CrO_4) (500 μg.)	0	—*
2:6-Di*tert.*-butyl-4-methylphenol (100 μg.)	25	—*
Catalase (about 0·1 Kiel unit)	76	13

* Yellow.

Table 3. *Effects of carbon monoxide on haemolysis and lipid peroxidation*

For details see text.

Treatment of vitamin E-deficient erythrocyte suspension	Haemolysis (%)	Thiobarbituric acid reaction (E at 550 mμ)
Nil	93	22
CO with CO atmosphere	12	7
CO with air atmosphere	38	16
CO with N_2 atmosphere (N_2 bubbled through the suspension)	7	7

protection of the erythrocytes *in vitro*. Catalase had little, if any, protective effect although enough was added to destroy about 100 mg. of hydrogen peroxide (100 vol.). No peroxides were found in vitamin E-deficient erythrocytes not treated with dialuric acid. Erythrocytes treated with carbon monoxide showed the cherry-red colour characteristic of carboxyhaemoglobin and were considerably resistant to haemolysis and peroxidation, especially in an atmosphere of carbon monoxide or nitrogen, the latter being passed through the solution to remove dissolved carbon monoxide. Exposure to air reduced resistance, owing probably to the reformation of oxyhaemoglobin (Table 3).

Erythrocyte fractions

Erythrocytes were haemolysed with water and separated into two fractions, the stroma and the stroma-free haemolysate. Each fraction was then treated with dialuric acid, with thiobarbituric acid to detect peroxidation. Peroxides were formed by intact vitamin E-deficient erythrocytes and their cell stroma, but not by the haemolysate unless

Table 4. *Effect of dialuric acid and thiobarbituric acid on erythrocytes and erythrocyte fractions*

Each fraction was equivalent to 2 ml. of 20 % erythrocyte suspension

	Haemolysis (%)	Thiobarbituric acid reaction (E at 550 mμ)
Normal erythrocytes		
Intact cells	0	—*
Stroma-free haemolysate	—	—*
Stroma	—	—*
Vitamin E-deficient erythrocytes		
Intact cells	90	14
Stroma-free haemolysate	—	—*
Stroma	—	7
Vitamin E-deficient erythrocytes pretreated with 10 μg. of α-tocopherol/2 ml.		
Intact cells	0	—*
Stroma-free haemolysate	—	—*
Stroma	—	—*
Vitamin E-deficient erythrocytes		
Haemolysate (centrifuged at 1000 *g*)	—	14

* Yellow.

Table 5. *Effect of* Fe^{2+} *and* Fe^{3+} *ions on vitamin E-deficient erythrocyte fractions treated with dialuric acid and thiobarbituric acid*

Fe^{2+} ions were as $FeSO_4,(NH_4)_2SO_4,6H_2O$ and Fe^{3+} ions as $FeCl_3,6H_2O$).

	Thiobarbituric acid reaction (E at 550 mμ)
Intact cells	17†
Stroma-free haemolysate	—*
Stroma	8
Stroma + haemolysate	17
Stroma in 2·5 mM-Fe^{2+}	8
Stroma in 2·5 mM-Fe^{3+}	19
Stroma in 2·5 mM-Fe^{2+} + 2·5 mM-Fe^{3+}	16
Stroma in 2·5 mM-Fe^{2+} (no dialuric acid)	—*
Stroma in 2·5 mM-Fe^{3+} (no dialuric acid)	—*

* Yellow. † 55 % haemolysis occurred.

Table 6. *Effect of* Fe^{2+} *and* Fe^{3+} *ions on rate of dialuric acid-induced haemolysis*

A 5 % vitamin E-deficient erythrocyte suspension was used.

Ion	Concentrations (mM)	Haemolysis (%) 10 min.	Haemolysis (%) 17 min.
Nil	Nil	47	81
Fe^{2+}	0·8	—	59
Fe^{2+}	0·5	42	69
Fe^{2+}	0·16	—	79
Fe^{3+}	0·5	11	56
Fe^{3+}	0·4	—	66
Fe^{3+}	0·8	—	41

this was prepared at 1000 *g*. No peroxides were found in normal erythrocytes, vitamin E-deficient erythrocytes fortified *in vitro* with α-tocopherol or their cell fractions (Table 4). Although itself inert, the haemolysate prepared at 20 000 *g* enhanced peroxidation of the stroma fraction (Table 5). Solutions of Fe^{2+} and Fe^{3+} ions were added to the stroma in amounts equivalent to the iron content of the haemolysate and the Fe^{3+} ion increased peroxide formation. Neither ion induced peroxidation of the stroma in the absence of dialuric acid.

Effects of Fe^{2+} *and* Fe^{3+} *ions on haemolysis*

Tests were carried out to see if Fe^{2+} or Fe^{3+} ions would accelerate haemolysis by dialuric acid (Table 6). An erythrocyte suspension (5 %) was used and concentrations of Fe^{2+} and Fe^{3+} ions up to 0·8 mM and 0·5 mM respectively were used (higher levels of Fe^{3+} ions resulted in erythrocyte sedimentation). No acceleration occurred; both ions, in fact, caused a partial delay in haemolysis.

Effects of dialuric acid on haemoglobin

The absorption spectrum of the pigment released from erythrocytes by haemolytic concentrations of dialuric acid appeared to be identical with that of oxyhaemoglobin when it was examined both by the direct-vision spectroscope and the spectrophotometer. Much higher concentrations of dialuric acid markedly affected the colour of vitamin E-deficient erythrocytes without causing haemolysis. When erythrocytes so treated were then haemolysed by dilution in water, the solution produced also appeared to contain only oxyhaemoglobin; but if the erythrocytes were first haemolysed in water, then treated by the addition of solid dialuric acid and further diluted for measurement, the type of pigment produced depended on whether this further dilution was with water or buffer pH 7·4 (Fig. 1). By comparison with standard curves (Hawk, Oser & Summerson, 1954) it was

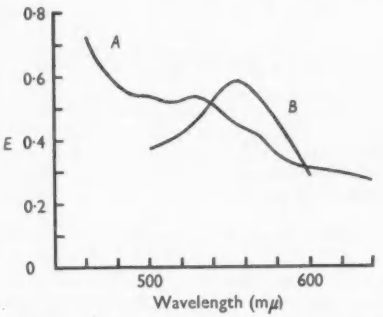

Fig. 1. Effect of excess of dialuric acid on vitamin E-deficient erythrocyte haemolysates, diluted in water (*A*), or buffer, pH 7·4 (*B*).

found that methaemoglobin was formed in water but haemoglobin was formed in buffer. It is possible therefore that haemoglobin may have been produced in the intact erythrocytes by the higher levels of dialuric acid, but re-oxidized by atmospheric oxygen on dilution with water.

Brückmann & Wertheimer (1945), investigating diabetogenic compounds, found that, *in vitro*, methaemoglobin was produced by alloxan, but that alloxantin and dialuric acid gave haemoglobin. The formation of methaemoglobin referred to here may therefore indicate oxidation of dialuric acid to alloxan in the unbuffered solution.

DISCUSSION

The present study shows a close parallel between dialuric acid-induced haemolysis and lipid peroxidation and demonstrates that agents which prevent haemolysis also stop peroxide formation. The theory of Rose & György (1950, 1952) that hydrogen peroxide itself is not the haemolytic agent is supported by the failure of catalase to prevent peroxidation, although these authors did find catalase active in their tests. Erythrocyte-fractionation experiments lead to the conclusion that the lipoprotein membrane itself and possibly some other material not firmly bound to the membrane is attacked by a product of dialuric acid.

A catalytic role for haemoglobin is suggested by the enhanced peroxide formation in stroma when haemolysate is added, an effect also given by Fe^{3+} but not by Fe^{2+} ions. Protection by carbon monoxide may be due to more than one effect; carboxyhaemoglobin is resistant to dissociation (but not to oxidation) and its formation in these tests involves liberation of oxygen from oxyhaemoglobin before addition of dialuric acid. If the formation of haemoglobin demonstrated in excess of buffered dialuric acid occurs to a small extent in haemolysing erythrocytes, a portion of the oxygen of oxyhaemoglobin may be released in the presence of dialuric acid, leading to the production of the intermediate postulated by Rose & György. In this case, as suggested by the same authors, excess of dialuric acid must also form a protective layer around the cell, so preventing haemolysis.

The enhancement of stroma peroxidation by Fe^{3+} ions is unexplained, but Kibrick, Safier & Skupp (1959) reported that thiobarbituric acid in the presence of 0·12 mM-ferric chloride detected peroxides even in normal blood.

SUMMARY

1. Dialuric acid-induced haemolysis of vitamin E-deficient erythrocytes has been shown to be accompanied by lipid peroxidation. Formation of peroxides is prevented by agents protecting erythrocytes against haemolysis (α-tocopherol, 2:6-di*tert.*-butyl-4-methylphenol and the ions Co^{2+}, Mn^{2+}, $Cr_2O_7^{2-}$ and CrO_4^{2-}). The protection of α-tocopherol is completely opposed by SeO_3^{2-} ions.

2. Studies with L-thyroxine have shown it to be fully protective *in vitro* against both haemolysis and lipid peroxidation.

3. Haemoglobin catalysis of lipid peroxidation in the erythrocyte stroma has been demonstrated by cell-fractionation studies and by the protection of intact cells resulting from the conversion of oxyhaemoglobin into carboxyhaemoglobin. From the observed effects of excess of dialuric acid, it seems that oxyhaemoglobin may dissociate during haemolysis, but only to a small extent.

REFERENCES

Bernheim, F., Bernheim, M. L. C. & Wilbur, K. M. (1947). *J. biol. Chem.* **174**, 257.
Brückmann, G. & Wertheimer, E. (1945). *Nature, Lond.*, **155**, 267.
Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T. (1960). *Biochem. J.* **75**, 460.
Carpenter, M. P., Kitabchi, A. E., McCay, P. B. & Caputto, R. (1959). *J. biol. Chem.* **234**, 2814.
Glavind, J. & Hartmann, S. (1951). *Acta chem. scand.* **5**, 975.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1954). *Practical Physiological Chemistry*, p. 473. London: J. and A. Churchill Ltd.
Horwitt, M. K., Harvey, C. C., Duncan, G. D. & Wilson, W. C. (1956). *Amer. J. clin. Nutr.* **4**, 408.
Kibrick, A. C., Safier, L. B. & Skupp, S. J. (1959). *Proc. Soc. exp. Biol., N.Y.*, **101**, 137.
Landucci, J. M., Pouradier, J. & Duranté, M. (1955). *C.R. Soc. Biol., Paris*, **149**, 919.
Patton, S. & Kurtz, G. W. (1951). *J. Dairy Sci.* **34**, 669.
Rose, C. S. & György, P. (1950). *Blood*, **5**, 1062.
Rose, C. S. & György, P. (1952). *Amer. J. Physiol.* **168**, 414.
Tappel, A. L. & Zalkin, H. (1959). *Arch. Biochem. Biophys.* **80**, 326.
Tappel, A. L. & Zalkin, H. (1960). *Nature, Lond.*, **185**, 35.
Tauber, H. & Petit, E. L. (1952). *J. biol. Chem.* **195**, 703.

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Changes in Enzymes of the Uterus of the Ovariectomized Rat after Treatment with Oestradiol

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The enzymes involved in the oxidation of glucose 6-phosphate are the dehydrogenases for glucose 6-phosphate and phosphogluconate, both of which require triphosphopyridine nucleotide as a coenzyme. In *Escherichia coli*, the extractable activity of these enzymes was found to be remarkably constant under certain conditions and could be induced to decrease or increase under other conditions. When the cells were grown in synthetic medium in which ammonium ions supplied nitrogen and only the carbon source was an organic compound, there was no significant difference in enzyme activities, between cells which were resting or growing, under aerobic or anaerobic conditions, or with several different carbon sources, such as glucose, gluconate or lactate (Scott, 1956*a*). The ratio of glucose 6-phosphate dehydrogenase to phosphogluconate dehydrogenase was $1{\cdot}9 \pm 0{\cdot}4$. However, when *E. coli* were grown in enriched medium, supplying preformed cell constituents, such as nutrient broth or casein hydrolysate, but not yeast ribonucleic acid, the glucose 6-phosphate-dehydrogenase activity was decreased whereas the phosphogluconate dehydrogenase was essentially unchanged and the ratio fell as low as 0·70 (Scott & Chu, 1959).

On the other hand, treatment with certain drugs, such as 2:4-dinitrophenol (Scott, 1956*b*) and nitrated benzimidazoles (Scott, Rogers & Rose, 1958), increased the glucose 6-phosphate dehydrogenase. The increase of activity was proportional to the concentration of the drug up to concentrations which inhibited growth. The increase was proportional also to the time of exposure to the drug. Again the activity of phosphogluconate dehydrogenase was not changed or was increased slightly so that ratios could more than double within 30 min. of growth in the presence of drug and could attain values as high as 12, i.e. six times the ratio of enzyme activities in synthetic medium without drug.

In animal tissues, Glock & McLean (1954) found evidence that these enzymes increased in the mammary gland during lactation. Glock & McLean (1955*b*) found a decrease in the phosphogluconate-dehydrogenase activity in the livers of alloxan-diabetic rats and increases of both dehydrogenases after insulin. An increase of these enzymes in livers of rats treated with thyroxin was reported by Glock, McLean & Whitehead (1956) and by Spiro & Ball (1958). Tepperman & Tepperman (1958) reported increases up to 15-fold in glucose 6-phosphate-dehydrogenase activity in the livers of rats which were fed with a high carbohydrate diet for 48 hr., after starvation for 48 hr. The liver glucose 6-phosphate-dehydrogenase activity was also increased after changing the rats' diet from normal mixed to high carbohydrate, without a period of starvation. Weber & Cantero (1957) have found that glucose 6-phosphate dehydrogenase is about five times as high in Novikoff hepatoma as in normal rat liver. Thus this constitutive enzyme would seem to be adaptively responsive in the mammal as well as in bacteria to changes in nutrition, to changes in hormone balance, and to whatever constitues the change from normal to tumour tissue.

In order to investigate the induction of changes in enzyme activity when a normal tissue changes from a maintenance state to an actively growing state, we chose a mammalian organ which grows under the influence of a steroid hormone and regresses when the hormonal effect wears off: the rat uterus under the influence of oestrogen.

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (disodium salt; ATP), triphosphopyridine nucleotide (monosodium salt; TPN), and reduced triphosphopyridine nucleotide (tetrasodium salt, tetrahydrate; TPNH, 90% pure) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Diphosphopyridine nucleotide (sodium salt; DPN) and reduced diphosphopyridine nucleotide (disodium salt; DPNH) were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. Glutathione and oxidized glutathione were obtained from Schwarz Laboratories Inc., Mt. Vernon, N.Y., U.S.A.

Glucose 6-phosphate (G 6-P) and phosphogluconate were the barium salts, kindly prepared by Dr M. L. Lanning by the method of Seegmiller & Horecker (1951). Solutions of the sodium salts were prepared by precipitation of the barium with sodium sulphate.

Oestradiol-17β was kindly supplied by the Schering Corp., Bloomfield, N.J., U.S.A.

Operation. The rats were Wistar-strain adult females obtained from the Wistar Institute and weighed between 140 and 240 g. They were maintained on Purina Dog Chow. Ovariectomy was accomplished under ether anaesthesia. A midline dorsal incision was made, the skin was retracted to the side and the ovary was extracted through a small, lateral incision on each side as described by Griffith & Farris (1942). A ligature was placed near the end of each horn of the uterus, which was then sectioned distally. Thus complete removal of the ovary was assured by inclusion of the Fallopian tube and a small section of the horn.

Injection. Oestradiol (1 mg./ml.) was dissolved in 95% ethanol. For injection, 0·1 ml. was drawn into a 1 ml. syringe, which was then filled to 1 ml. with sterile 0·9% NaCl solution. This 100 μg. was injected into the peritoneum of the rats under light ether anaesthesia, 10 days after the operation.

Preparation of extracts. At selected times after the injection, the animals were killed by cervical concussion. After the uterus was exposed and its gross appearance noted, it was quickly excised at the fundus. The two horns and the small section of fundus were first freed of fat and connective tissue and then washed with NaCl solution, blotted and weighed. The piece was placed on a dry Petri dish and the horns were slit lengthwise with scissors. The endometrial surface was flushed with 0·5 ml. of ice-cold 0·25 M-sucrose solution and scraped with a scalpel to remove as much of the endometrium as possible. The scrapings were washed and collected in additional cold sucrose solution to a total volume of 1·0–1·5 ml. The suspension of broken cells (10–30 mg. dry wt.) was placed in a centrifuge tube in an ice bath without homogenizing.

The myometrium was blotted and weighed. The tissue was cut finely with scissors and placed in a steel chamber which had been chilled in the freezer at $-20°$. A similarly chilled steel plunger was fitted on to a collar around the tissue and hit repeatedly with a large hammer. The fragmented frozen tissue was then homogenized in a small Elvehjem tube with a Teflon plunger with 10 vol. of ice-cold sucrose solution for 5 min. The tube was immersed in an ice bath throughout the homogenization.

When the adrenals were also analysed they were removed before the uterus and placed in the freezer while the uterus was being processed. After being cleaned, the adrenals were weighed and homogenized with approx. 2 ml. of cold sucrose solution in a small glass hand homogenizer for 2 min.

The homogenates and the suspension of endometrial scrapings were centrifuged for 1 hr. in an International refrigerated centrifuge at 2200 *g*. The supernatant extract contained not only the soluble protein but also the small particles, probably most of the microsomes. The dehydrogenases studied have not been found to act differently from those of extracts centrifuged at 10 000 *g* for 1 hr. The particulate residues were dried at 105° to constant weight.

These enzymes are unstable and may lose activity during preparation, especially during the longer procedure for the myometrium. This was minimized by maintaining a low but not freezing temperature and made uniform by standardizing the procedures as much as possible. Frozen extracts lost from 40 to 70% of the glucose 6-phosphate-dehydrogenase activity in 4 days. The loss in activity for glucose 6-phosphate dehydrogenase at 4° was about 15–30% in 2–4 hr. Backward extrapolation shows that it is possible that 10–15% of activity was lost during preparation of the extracts. The dehydrogenase activities of extracts which had been frozen or had been standing at 4° for several hours could be partially reactivated by the addition of bovine-serum albumin to the mixture. The addition of albumin had little effect on the activity of fresh extracts. The addition of cysteine either during homogenizing or to the reaction mixtures had a variable effect, usually inhibitory.

Enzyme determinations

Dehydrogenases. Enzyme activities of the extracts were determined immediately. The dehydrogenase activities in terms of rate of reduction of TPN at 35° were measured by increase of absorption at 340 mμ in a Beckman spectrophotometer. Samples of the supernatant fluids chosen to give an increase of extinction of 0·010–0·050/min. were added to 1 ml. cuvettes containing 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4, and 0·5 μmole of substrate, either glucose 6-phosphate or phosphogluconate, in double-distilled water to give a final volume of 1 ml., which had been brought to 35°. Addition of Mg^{2+} ions was not necessary. TPN solution (0·3 mg.) was added and readings were taken at 0·5 min. intervals for 5 or 6 min. In blanks with no added substrate the reduction of TPN by endogenous substrate was found to be almost finished in the first minute except by the most active extracts. Therefore, except in the last-named cases, no correction was made for endogenous reduction of TPN. The glucose 6-phosphate-dehydrogenase activities were not corrected for reduction of TPN by dehydrogenase acting on the resulting phosphogluconate, for the maximum concentration of phosphogluconate produced in the first 5 min. was less than 0·04 mM, a concentration at which the dehydrogenase has less than half the maximum activity. Therefore the rates of both enzymes were chosen as the mean increase in extinction/min. between the first and fourth minutes.

Results are reported as change in extinction at 340 mμ/min./mg. of protein, or have been converted into rates in μmoles of TPN reduced/min./mg. of protein by division by 6·22 (Horecker & Kornberg, 1948).

The difference between duplicate determinations of glucose 6-phosphate dehydrogenase was usually less than 10% unless the time between the duplicates was greater than 2 hr. The difference between duplicate phosphogluconate-dehydrogenase determinations was less than that of the other dehydrogenase. The second determination was the lower one except when the sample size of the first was below the set limits of activity. Because of the loss of activity with time the results recorded here are the first determinations within the set limit.

Determinations of dehydrogenase activities and protein content were performed on all the extracts. Hexokinase, transhydrogenase and/or TPNH-oxidase activities were determined whenever there was sufficient extract remaining.

Transhydrogenase. Transhydrogenase was determined by the methods of Kaplan, Colowick & Neufeld (1953) and Talalay & Williams-Ashman (1958). Fig. 1 illustrates the measurement of the transhydrogenase reaction of extracts of the adrenal and myometrium and the absence of it in the

endometrial extract of rat no. 51. The transhydrogenase activity was calculated as the increase in $E_{340\,m\mu}$/min./mg. of protein due to production of DPNH after all the TPN was reduced. Insufficient material was available in this series for a study of the transhydrogenase activity of the unfractionated homogenate.

Hexokinase. Hexokinase of the extracts was determined by either the direct or the indirect method or both (Slein, Cori & Cori, 1950). By the first method samples of the extracts containing approx. 0·5 mg. of protein were added to cuvettes containing 1·1 μmole of glucose, 1 μmole of Mg^{2+} ions and 1 μmole of ATP in 1 ml. of 0·05 M-tris buffer, pH 7·4. After 10 min. 0·4 μmole of TPN was added and from the curve of TPNH formation the amount of glucose 6-phosphate which had been formed was calculated with corrections for endogenous substrate and rate of glucose 6-phosphate formation. By the direct method, more commonly employed, the extract was added to cuvettes containing 1·1 μmole of glucose, 1 μmole of $MgSO_4$, 0·4 μmole of TPN and 0·05 M-tris buffer in a total volume of 1 ml. After reduction, caused by endogenous ATP and substrate, had become minimal, 1 μmole of ATP was added and the rate of reduction of TPN determined. In these determinations the glucose 6-phosphate dehydrogenase of the extracts was considered to be not rate-limiting. No attempt was made to determine the hexokinase activity either of the whole homogenate or of the particulate fraction of the homogenates.

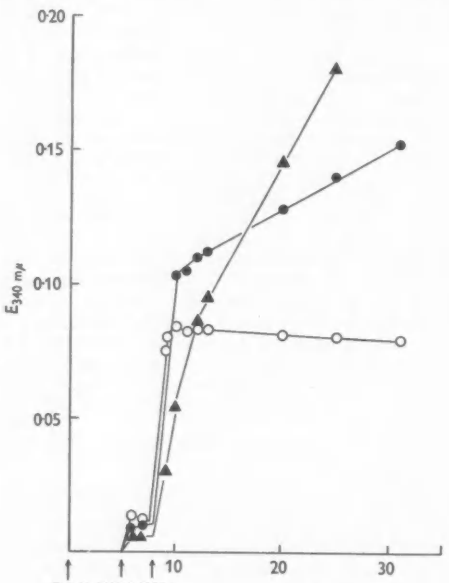

Fig. 1. Transhydrogenases of extracts of rat uterus. Rat no. 51 received 100 μg. of oestradiol 10 days after ovariectomy and again 24 hr. later and was killed after a further 24 hr. Each 1·5 ml. cuvette contained 5 μmoles of glucose 6-phosphate (G 6-P) in tris buffer, 0·05 M, pH 7·4. Extracts, in 0·25 M-sucrose, were: ▲, 0·02 ml. of adrenal gland (40 μg. of protein); ○, 0·1 ml. of endometrial (116 μg. of protein); ●, 0·05 ml. of myometrial (150 μg. of protein). At 5 min. 0·02 ml. of DPN (10 mg./ml.) and at 8 min. 0·01 ml. of TPN (1 mg./ml.) were added to each cuvette. Total volume was 1 ml. Extinction $E_{340\,m\mu}$ was read on a Beckman spectrophotometer at 35°. Complete reduction of the TPN added to the system would give an increase in $E_{340\,m\mu}$ of about 0·08.

Reduced triphosphopyridine nucleotide oxidase. To a cuvette, containing 1 ml. of 0·05 M-tris, pH 7·4, and 0·06 ml. of extract at 35°, was added 0·02 ml. (0·116 mg.) of freshly prepared TPNH. The decrease in $E_{340\,m\mu}$ during 10–20 min. was compared with the non-enzymic decrease in a parallel cuvette. Then 0·2 μmole of oxidized glutathione was added and the readings were continued for 10–20 min. further to determine TPNH-glutathione reductase. Both TPNH oxidases were slightly inhibited by either Mg^{2+} ions or Mn^{2+} ions at a concentration of 4 mM.

Other determinations. TPN nucleotidase was determined by the cyanide method of Kaplan *et al.* (1953).

Amperometric titrations of acid-soluble SH groups were performed by the method of Benesch, Lardy & Benesch (1955).

The protein content of the extracts was determined by the bromsulphalein method of Bonting & Jones (1957), with bovine-serum albumin as a standard.

RESULTS

Results are reported on 37 rats. Two rats died during the operation and they were counted as unoperated controls.

Table 1 presents data on the weights of the animals (*c*), the wet weight of the whole uterus (*d*) and the scraped uterus (*e*), the dry weight of the extracted scraped uterus (homogenate residue *g*) and the total soluble protein in the myometrial extracts (*j*). There was no correlation between the uterine weight and the weight of the whole rat. The wet weight of the uterus (*d*) was increased after 2 hr. after oestradiol, over the control ovariectomized rat. An increase was not evident in the dry weight (*g*). This is in line with the findings of others (Reynolds, 1949) that there is imbibition of water by the uterus of the ovariectomized rat, at about 2 hr. after the injection of oestrogens. That this increase of water content occurred in the myometrium as well as the endometrium is shown by column *h*, the percentage dry weight of the scraped uterus. The change in mass after scraping is calculated in column *f*. This value does not seem to correspond to the weight of endometrial tissue scraped off, for the sum of the dry weight of the endometrial residue plus the total protein of the extract ranged between 12 and 22 mg. Column *f* would seem to indicate that the control uteri imbibed water *in vitro* from the sucrose solution during scraping. Uteri, removed in the period

Table 1. *Effect of oestradiol on weights and soluble protein of uteri of ovariectomized rats*

(*a*) Animal no.	(*b*) Time of killing after oestradiol (hr.)	(*c*) Wt. of rat (g.)	(*d*) Whole uterus (mg. wet wt.)	(*e*) Scraped uterus (mg. wet wt.)	(*f*) Difference (*e*) − (*d*) (mg.)	(*g*) Homogenate residue (mg. dry wt.)	(*h*) Percentage dry wt. [100 (*g*)/(*e*)]	(*j*) Total soluble protein of myometrium (mg.)	(*k*) Percentage of soluble protein [100 (*j*)/(*g* + *j*)]
43	0	180	146	159	+13	62	39	4·5	6·8
44	0	182	125	142	+17	61	43	4·9	7·3
48	0	184	172	179	+ 7	61	34	4·1	6·3
37	2	204	158	146	−12	45	31	5·5	10·9
31	2·2	224	213	203	−10	64	31	5·6	8·0
29	2·5	202	273	209	−64	64	30	5·9	8·4
39	4·2	200	275	240	−35	64	27	11·4	15·1
32	4·3	211	258	234	−24	75	32	5·9	7·3
33	6	187	253	216	−37	56	26	7·5	11·8
41	8·2	227	350	288	−62	77	27	9·0	10·5
34	8·2	231	322	263	−59	81	31	6·5	7·4
42	10·2	199	303	261	−42	59	23	11·0	15·7
26	12·4	191	—	203	—	59	29	5·4	8·4
49	15	180	321	281	−40	77	27	8·1	9·5
46	15·3	185	226	209	−17	64	31	4·1	6·0
38	18	242	206	198	− 8	51	26	7·3	12·4
35	24	210	182	181	− 1	60	33	8·2	12·0
47	36	181	214	203	−13	63	31	6·5	9·4
36	48	202	239	234	− 5	69	29	9·6	12·2
30	44·3	237	322	303	−19	93	31	9·7	9·4
28	76	241	191	191	0	81	42	5·6	6·5
51	48*	188	269	267	− 2	84	31	5·5	6·2
52	48*	186	256	244	−12	75	31	7·1	8·7

* Two injections.

2·5–15 hr. after oestradiol, lost water as well as endometrial tissue during scraping.

Column *k* is the percentage of soluble protein of the extracts of the myometrium, with the dry weight plus the soluble protein as 100%. This value was below 7·5% for control uteri, and except for rat no. 46, from 7·3 to 15% for at least 48 hr. after one injection of oestradiol. Some or all of this increase may represent the uptake of serum proteins (Kalman & Lowenstein, 1958).

Dehydrogenases. Table 2 gives the dehydrogenase activities of extracts of the endometria of 31 ovariectomized rats, 26 of which had been injected with oestradiol and five of which were killed as controls. The calculated specific activities are charted in Fig. 2. A biphasic pattern of change in the enzyme activities is evident. Both enzyme activities were decreased from 2 to 10 hr. after oestradiol as compared with control. From this low point of enzyme activity, the glucose 6-phosphate-dehydrogenase activity started to rise at 12 hr., and at 36–48 hr. attained activity about three times that of the ovariectomized control uteri and then decreased. The phosphogluconate-dehydrogenase activity also fell at 2 hr., started to rise at 12 hr. and achieved a maximum with one injection of oestradiol between 36 and 48 hr. Two injections of oestradiol at 24 hr. intervals increased the 48 hr. enzyme activities over those with one injection.

Extracts of myometrium exhibited no early decrease of dehydrogenase activity (Fig. 3). Increase in glucose 6-phosphate-dehydrogenase activity was evident at 15 hr.; there was a twofold increase by 36 hr. and the activity had not returned to normal in the one uterus analysed at 76 hr. With one injection of oestradiol, the change in the phosphogluconate-dehydrogenase activities was significant at the 5% level at 15–24 hr. and at the 1% level at 36 hr. With two injections, both enzymes were more markedly increased at 48 hr.; the phosphogluconate dehydrogenase was three and four times the mean control activity, and glucose 6-phosphate dehydrogenase was increased three- to six-fold.

Since these activities were calculated in terms of increase of absorption at 340 mμ due to the reduction of TPN/mg. of protein, the drop in activity in the first 2 hr. may have been caused by increased protein content due to serum protein leaking into the uterus. Therefore the total dehydrogenase activities of the extract were divided by the 'dry weight' of the uterine residue, that is of the homogenates from which the soluble protein had been extracted. Both series were analysed statistically. The values for the enzyme activities seemed to fall

Table 2. *Dehydrogenase activities of extract of uterus*

G 6-P, glucose 6-phosphate; PG, phosphogluconate; ΔE, change in $E_{340\ m\mu}$

		Myometrium				Endometrium			
				Dehydrogenase activities		Extract		Dehydrogenase activities	
Rat no.	Time after oestradiol (hr.)	Homogenate vol. (ml.)	Extract protein (mg./ml.)	G 6-P (ΔE/min./ml.)	PG (ΔE/min./ml.)	Vol. (ml.)	Protein (mg./ml.)	G 6-P (ΔE/min./ml.)	PG (ΔE/min./ml.)
16	0	—	2·3	0·55	0·26	—	0·50	0·25	0·103
17	0	—	1·6	0·53	0·18	—	0·88	0·30	0·106
40	0	2·0	4·40	0·85	0·40	1·2	1·03	0·44	0·20
43	0	1·8	2·72	0·90	0·54	1·3	0·37	0·32	0·16
44	0	1·8	2·96	0·84	0·48	1·6	0·35	0·25	0·11
37	2	2·4	2·38	0·58	0·30	1·5	0·68	0·06	0·09
31	2·2	2·1	2·96	0·96	0·56	0·9	1·00	0·38	0·15
29	2·5	2·2	2·96	1·00	0·60	1·2	1·00	0·25	0·10
39	4·1	2·0	6·46	0·90	0·50	1·4	2·67	0·48	0·22
32	4·3	2·0	3·26	0·92	0·50	1·2	1·05	0·29	0·11
25	6	1·9	1·95	0·70	0·20	1·3	0·68	0·24	0·08
33	6	2·3	3·60	0·80	0·44	1·2	1·22	0·23	0·12
34	8·2	2·0	3·80	1·34	0·78	1·2	2·50	0·58	0·25
41	8·2	2·2	4·76	1·20	0·60	2·4	2·68	0·78	0·35
42	10·1	2·1	5·94	1·60	0·80	1·4	2·29	0·50	0·25
26	12·4	1·8	3·39	1·00	0·48	—	0·92	0·52	0·19
49	15·0	2·1	4·46	2·10	1·00	1·2	1·68	1·20	0·55
46	15·2	1·6	2·97	1·56	0·60	1·5	0·99	0·73	0·29
22	18	—	2·12	0·96	0·50	—	0·41	0·20	0·06
23	18	—	2·60	1·34	0·67	—	0·72	0·60	0·18
38	18	2·1	3·84	1·14	0·52	1·7	0·95	0·50	0·20
35	24	2·3	3·93	1·20	0·52	1·3	0·56	0·13	0·06
45	24·3	—	—	—	—	1·2	1·25	1·35	0·50
47	36	1·7	4·30	3·20	1·30	0·9	1·14	2·46	0·90
50	39	2·0	4·40	2·55	0·93	1·0	1·46	2·45	0·68
24	40	—	3·60	2·00	0·92	—	1·05	1·70	0·60
30	44·3	2·5	4·43	2·60	1·25	1·1	1·03	2·20	0·65
36	48	2·2	4·81	2·40	1·00	1·2	0·93	1·25	0·45
28	76	2·3	2·68	1·35	0·40	1·3	0·65	0·49	0·20
51	48 (2×)	2·1	2·98	2·86	1·42	1·1	1·16	2·40	0·95
20	48 (2×)	—	2·18	2·55	1·50	—	0·62	1·56	0·52
21	48 (2×)	—	1·70	3·0	0·80	—	0·65	2·2	0·63

in periods for which means were determined and the significance of the differences between them were calculated as shown in Table 3. In Table 3 the drop in specific activity of glucose 6-phosphate dehydrogenase of endometrial extracts in the period 2–10 hr. after oestradiol was seen to be significant. However, when calculated in terms of total activity per 'dry weight' the decrease was not significant (Table 4).

Hexokinase. Activators, or inhibitors of other reactions, such as nicotinamide, ethylenediaminetetra-acetate (EDTA) or potassium fluoride have been found not to affect the activity of the dehydrogenases in fresh extracts of two other mammalian tissues which have been studied subsequent to the work on the uterus, i.e. hamster cheek pouch and cultured rabbit-kidney cells. Hexokinase activity was increased by EDTA and potassium fluoride and these effects were not additive. Also for the hexokinase determination, where the reactions continue over a longer time, nicotinamide was found necessary to protect the TPN from TPN nucleotidase. Samples of extracts of endometrium, myometrium and adrenal gland were tested for TPN nucleotidase and destruction of TPN was found to be not greater than 2% in 10 min. at 37°. None of the other activators were used in the determination of uterine hexokinase, because the uterine extracts available were not sufficient for an adequate study of their effects. These results are so incomplete for this series that they are presented only as suggesting that the activity of this enzyme may also be affected by injection of oestradiol and that the effect deserves further investigation.

The series of hexokinase determinations on the extracts of both the endometrium and the myometrium suggested a biphasic response of the endometrial enzyme, as shown in Table 5. In the endometrial extract the increase with two injections of oestradiol was only 100% at 48 hr. The hexokinase of myometrial extracts increased 100% at 36 hr. after one injection and 200% at 48 hr. after two injections of oestradiol.

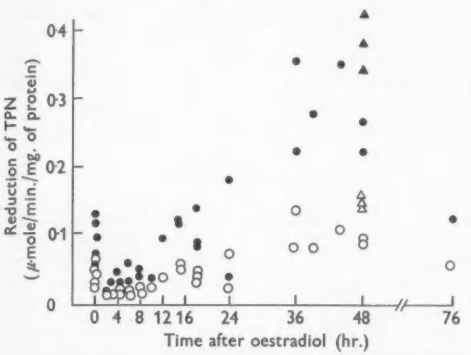

Fig. 2. Dehydrogenase activities of extracts of endometrium. Each cuvette contained 0·05 M-tris buffer, pH 7·4, and 1 μmole of glucose 6-phosphate (●) or 0·5 μmole of phosphogluconate (○) and 0·1 or 0·2 ml. of extract in a total volume of 1 ml. The extract was the supernatant of the scrapings of the endometrial surface of the uterus in 0·25 M-sucrose and contained 0·5–3·0 mg. of protein/ml. TPN, 0·02 ml. (200 μg.), was added and $E_{340\,m\mu}$ was read at 0·5 min. intervals for 5 min. The enzyme activity was calculated from the change in $E_{340\,m\mu}$/min. from the first to fourth minutes to μmoles of TPN reduced/min./mg. of protein. Each symbol represents the enzyme activity of the extract of one rat. Ten days after ovariectomy, controls were killed and treated rats were injected with 100 μg. of oestradiol and killed at a time later as shown on the abscissa. △ and ▲, the rat had received a second injection of 100 μg. of oestradiol at 24 hr. and was killed at 48 hr.

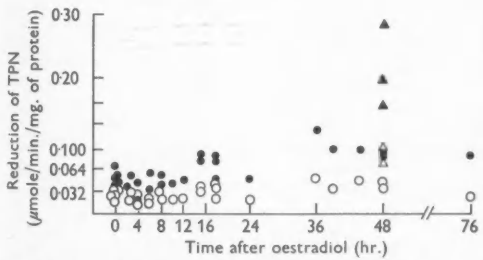

Fig. 3. Dehydrogenase activities of extracts of myometrium. Enzyme activities were determined as in Fig. 2, with 0·05 or 0·1 ml. of extract of the scraped homogenized uterus, containing 100–200 μg. of protein. ●, Glucose 6-phosphate dehydrogenase; ○, phosphogluconate dehydrogenase. △ and ▲, See legend to Fig. 2.

Transhydrogenase. Determination of transhydrogenase on the extracts of the uterus revealed no activity in the endometrial extracts and moderate transhydrogenase in the myometrial extracts. Values for the transhydrogenase of myometrial extract were decreased after oestradiol injection and recovered after 10 hr. (Table 5). These values may be compared with the active transhydrogenase in extracts of adrenal gland (Table 7). Addition of oestradiol to the myometrial extracts of ovariectomized controls did not increase the activity.

Endogenous substrates. The reduction of TPN by endogenous substrates during the first minute is shown in Table 6. The most definite pattern in the endometrium is evident in the endogenous substrate calculated as μmole/mg. of protein in the extract. The amounts decreased at 2 hr. compared with the control and increased at 12·5–24 hr., decreasing to the control level later. The myometrial extracts from control rats contained very little endogenous substrate/mg. of protein. From 2 to 48 hr. after oestradiol there was a steady increase and at 76 hr. the values had not declined to levels as low as the controls. We have not obtained sufficient material to enable us to determine the identity of this endogenous substrate which reacts with TPN.

Reduced triphosphopyridine nucleotide oxidases. Samples of extracts of the uteri of two intact rats were analysed for glutathione. Direct titration of the extracts from which protein had been precipitated by 5% sulphosalicylic acid gave mean values of 0·050 and 0·049 μmole of soluble SH group/mg. of protein for the endometrium and the corresponding values of 0·045 and 0·036 for the myometrium. When the extracts were treated with TPN and glucose 6-phosphate before the protein was precipitated, the titrations indicated 0·062 and 0·055 μmole/mg. of protein for the endometrium and myometrium respectively. Thus in preparation of endometrium extract 21% of the SH groups were oxidized. In the homogenized myometrial extract 35% of the SH groups were oxidized. If all of the glutathione were to be oxidized, the amount in a sample of aged extract, containing 0·05–0·2 mg. of endometrial protein, would contain not more than 0·01 μmole of oxidized glutathione. Samples of freshly prepared endometrial extracts used for enzyme assay might be expected to contain up to 0·002 μmole of oxidized glutathione, which in oxidizing TPNH might cause a total decrease in extinction of 0·012, which is negligible. Similar calculations for oxidized glutathione of samples of myometrial extracts might cause a total change of 0·036 in extinction. The rate of oxidation of TPNH by glutathione would extend this reaction over a longer period than that taken for dehydrogenase measurement and therefore its effect would be negligible.

Freshly prepared extracts which had been kept cold did not reoxidize TPNH after the substrate was exhausted unless oxidized glutathione was added to the reaction mixture. Tests performed with extracts of endometrium and myometrium

from operated rats at 12 hr. and 48 hr. after injection of oestradiol oxidized no TPNH even in the presence of oxidized glutathione.

Fig. 4 is the graph of an experiment on the oxidation of added TPNH by fresh extracts of the endometrium and the myometrium of the uterus of an unoperated rat and the control oxidation in the tris buffer without extract. The oxidation of TPNH by extracts is only slightly greater than without them and is not much increased by addition of 0·2 μmole of oxidized glutathione, an amount greater than is present in the tissue extracts. These rates are too low to have any influence on the rates of reduction of TPN by the dehydrogenases.

With limiting concentration of TPN, either *in vitro* or *in vivo*, TPNH oxidation by a dye (Dickens & McIlwain, 1938) or some other hydrogen acceptor (Kinoshita, 1957) is necessary for the continued availability of TPN for the dehydrogenase reactions. In our assay with substrate concentrations of TPN the rate is not limited by TPN concentration nor inhibited by the TPNH formed, in the first few minutes.

Adrenal-gland enzymes. The enzyme activities of the extracts of the adrenals (Table 7) varied considerably, with no correlation with time after oestradiol injection. All extracts tested had high transhydrogenase activity. No endogenous substrate was found that reduced TPN.

DISCUSSION

The dehydrogenase, but not the hexokinase, activities as measured by the assays represent the near maximum potential activity of the enzymes *in vitro*. This potential would not be expected to be fully expressed *in vivo* unless conditions were such that both the substrate was present in optimum concentration and sufficient TPN was present in the oxidized state. Analyses of other normal tissues have indicated that glucose 6-phosphate concentration is suboptimum, phosphogluconate has not been detected and, according to Glock & McLean (1955*a*), TPN is more than 90 % reduced except in muscle and the total in liver is about equal to the Michaelis constant (10^{-6} M). Calculations of the

Table 3. *Specific dehydrogenase activities*

For G 6-P, PG and ΔE see Table 2. Numbers of rats are given in parentheses.

		Myometrial extracts	
		G 6-P (ΔE/min./mg. of protein)	PG (ΔE/min./mg. of protein)
Controls	Mean	(6) 0·30	(6) 0·145
	S.D.	0·080	0·045
2–12 hr.	Mean	(11) 0·29	(10) 0·147
	S.D.	0·069	0·035
Combined 0–12 hr.	Mean	(17) 0·29	(16) 0·146
	S.D.	0·067	0·040
15–24 hr.	Mean	(6) 0·43	(6) 0·20
	S.D.	0·10	0·052
	P (*vs.* control)	<0·01	<0·05
36–48 hr.	Mean	(5) 0·60	(5) 0·25
	S.D.	<0·094	0·041
	P (*vs.* control)	<0·01	<0·01
		Endometrial extracts	
		G 6-P (ΔE/min./mg. of protein)	PG (ΔE/min./mg. of protein)
Controls	Mean	(5) 0·57	(5) 0·24
	S.D.	0·22	0·12
2–10 hr.	Mean	(10) 0·23	(9) 0·137
	S.D.	0·096	0·08
	P (*vs.* control)	<0·01	>0·05*
12–24 hr.	Mean	(8) 0·63	(8) 0·25
	S.D.	0·24	0·095
	P (*vs.* control)	>0·05*	>0·05*
	P (*vs.* 2–10 hr.)	<0·01	<0·05
36–48 hr.	Mean	(5) 1·79	(5) 0·58
	S.D.	0·37	0·42
	P (*vs.* control)	<0·01	<0·01

* Difference is not significant.

Table 4. *Dehydrogenase activities calculated as total units/g. dry wt. of insoluble residue*

Numbers of rats are given in parentheses. For G 6-P, PG and ΔE see Table 2.

		Myometrial extracts	
		G 6-P (total ΔE/min./g. dry wt.)	PG (total ΔE/min./g. dry wt.)
Controls	Mean	(3) 32·2	(3) 17·3
	S.D.	3·8	4·2
2–8 hr.	Mean	(9) 30·3	(9) 16·1
	S.D.	3·6	4·1
	P (*vs.* control)	>0·05*	>0·05*
10–24 hr.	Mean	(7) 40·0	(6) 23·0
	S.D.	11·2	6·2
	P (*vs.* control)	>0·05*	>0·05*
	P (*vs.* 2–8 hr.)	<0·05	<0·05
	P (*vs.* combined 0–8 hr.)	<0·05	<0·05
36–48 hr.	Mean	(4) 78·0	(4) 33·0
	S.D.	6·6	1·8
	P (*vs.* control)	<0·01	<0·01
		Endometrial extracts	
		G 6-P (total ΔE/min./g. dry wt.)	PG (total ΔE/min./g. dry wt.)
Controls	Mean	(3) 9·2	(3) 4·2
	S.D.	4·5	2·0
2–6 hr.	Mean	(6) 6·0	(7) 2·5
	S.D.	2·2	1·05
	P (*vs.* control)	>0·05*	>0·05*
8–24 hr.	Mean	(8) 15·6	(8) 6·0
	S.D.	7·5	3·1
	P (*vs.* control)	>0·05*	>0·05*
	P (*vs.* 2–6 hr.)	0·01	<0·05
36–48 hr.	Mean	(4) 30·8	(4) 9·7
	S.D.	2·6	2·4
	P (*vs.* control)	<0·01	<0·05
	P (*vs.* 2–6 hr.)	<0·01	<0·01
	P (*vs.* 8–24 hr.)	<0·01	<0·05

* Difference is not significant.

Table 5. *Hexokinase and transhydrogenase activities of uterus extracts*

ΔE is change in $E_{340\ m\mu}$.

Rat no.	Time after oestradiol (hr.)	Hexokinase (ΔE/min./mg. of protein)		Transhydrogenase (ΔE/min./mg. of protein)	
		Endometrium	Myometrium	Endometrium	Myometrium
40	0	—	0·03	—	0·010
48	0	—	—	—	0·030
43	0	0·04	0·04	0	0·022*
31	2	—	—	0	0
39	4	—	0·017	0	0·002
41	8	0·017	0·025	0	0·005
42	10	0·010	0·04	0	0
46	15	0·05	0·05	—	0·017
49	15	0·024	0·05	0	0·022
47	36	0·035	0·075	0	0·022
50	39	—	—	0	0·013
51	48†	0·09	0·12	0	0·013

* Activity was the same when 10 μg. of oestradiol was added to the reaction mixture.
† Two injections of oestradiol.

Table 6. *Endogenous substrate for triphosphopyridine nucleotide in extracts of uterus*

The change in $E_{340\ m\mu}$ (ΔE) was measured during the first minute under the conditions described in the text.

Time after oestradiol (hr.)	Endometrium: Initial rate (ΔE/min./mg.)	Endometrium: Amount of TPN reduced: μmole/mg. of protein	Endometrium: Amount of TPN reduced: Total μmole	Myometrium: Initial rate (ΔE/min./mg.)	Myometrium: Amount of TPN reduced: μmole/mg. of protein	Myometrium: Amount of TPN reduced: Total μmole
0	0·09	0·060	0·074	>0	0·007	0·057
0	0·04	0·040	0·026	>0	0·009	0·044
0	0·08	—	—	>0	—	—
2	0·01	0·016	0·014	0·01	0·024	0·135
2·5	0·02	0·022	0·035	0·01	0·012	0·070
8	0·06	0·028	0·180	0·02	0·014	0·125
8·5	—	—	—	0·03	—	—
10	0·01	0·014	0·045	0·03	0·022	0·240
12·5	0·08	0·078	0·086	0·03	0·017	0·091
15	0·05	0·045	0·067	0·04	0·035	0·144
15	0·11	0·062	0·124	0·007	0·038	0·307
18	0·055	—	—	0·038	0·032	0·234
24	0·20	0·10	0·15	0·02	0·016	0·131
36	0·14	0·056	0·058	0·10	0·056	0·363
39	0·10	0·056	0·082	0·05	0·040	0·303
48	0·13	0·056	0·063	0·11	0·061	0·590
48*	0·21	0·113	0·145	0·13	0·069	0·380
76	0·046	0·040	—	0·041	0·023	0·129

* Two injections.

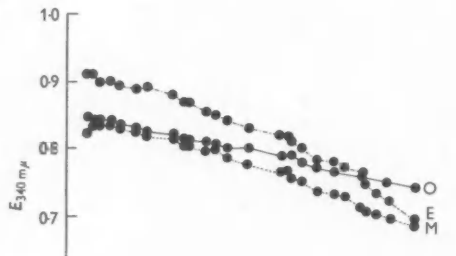

Fig. 4. Reduced triphosphopyridine nucleotide oxidase of extracts of rat uterus. Each cuvette contained 0·2 ml. of 0·05 M-tris, pH 7·4, and 0·02 ml. of TPNH (0·116 mg.). Total volume was 1 ml. O, Non-enzymic control; to E was added 0·06 ml. of extract of endometrium containing 66 μg. of protein; to M was added 0·06 ml. of extract of myometrium containing 132 μg. of protein. The uterus was taken from an intact rat. At 21 min. 0·01 ml. of oxidized glutathione (GSSG; 0·2 μmole of glutathione) and at 41 min. 0·01 ml. of cysteine (0·1 μmole) were added to all cuvettes. At 54 min. a further 0·06 ml. of endometrial extract was added to E and 0·12 ml. of myometrial extract was added to M.

total glucose 6-phosphate-dehydrogenase activity of extracts of *E. coli* have shown it to be sufficient to account for up to 80 % of the glucose used by a bacterial culture and the phosphogluconate dehydrogenase could account for about 40 % of the glucose (Scott & Cohen, 1953); isotope experiments have indicated that approx. 15–40 % of the glucose is so used by *E. coli* (Cohen, 1951; Roberts, Abelson, Cowie, Bolton & Britten, 1955). For other organisms and tissues, isotopic data have indicated similar or much smaller proportions of glucose metabolized via the hexose monophosphate shunt. Even if one considers the possibility that some of the carbon dioxide released from phosphogluconate may be recaptured by synthetic reactions, it is obvious that in *E. coli*, and probably in mammalian tissues (Beck, 1958), these dehydrogenases, especially the glucose 6-phosphate dehydrogenase, are not operating at full capacity.

When the normal or steady state is interrupted by a change in the environment that will increase the substrate, such as an increase in permeability or of hexokinase activity or the addition of a hydrogen acceptor, which will increase the TPN:TPNH ratio, then one may visualize a state in which the enzyme is operating at capacity. In such a state all the enzyme would be bound to substrate and if the substrate were still in excess one could expect new enzyme formation to be induced. Whether glucose 6-phosphate or TPN or both are the substrates which are necessarily bound is not clear. In the oestradiol-treated uterus, either in-

Table 7. *Activities of some enzymes in adrenal-gland extracts*

For G 6-P, PG and ΔE see Table 2.

Rat no.	Time (hr.)	Wet wt. (mg.)	G 6-P dehydrogenase (ΔE/min./mg. of protein)	PG dehydrogenase (ΔE/min./mg. of protein)	Hexokinase (ΔE/min./mg. of protein)	Transhydrogenase (ΔE/min./mg. of protein)
40	0	43	0·27	0·61	0·14	0·14
43	0	22	2·5	2·6	0·10	0·20
44	0	23	1·82	1·32	—	—
37	2	32	0·75	0·73	—	—
31	2·2	54	0·65	0·65	—	0·16
29	2·5	53	0·95	0·93	—	—
39	4·2	—	0·90	0·90	0·033	0·24
32	4·3	55	1·3	1·0	—	—
34	8·2	32	1·07	1·07	—	—
41	8·2	53	0·70	0·92	—	—
42	10·2	58	1·27	1·07	0·04	—
26	12·4	42	0·90	0·76	—	0·15
46	15·3	53	1·19	1·31	—	—
49	15	43	1·31	1·54	0·06	0·20
38	18	46	0·74	0·85	—	—
35	24	56	1·14	0·91	—	—
45	24·3	47	2·02	3·14	0·032	—
47	36	43	0·78	0·85	0	0·30
50	39	60	1·62	1·67	—	0·25
24	40	41	0·55	0·65	—	—
30	44·3	64	0·86	0·92	—	—
36	48	47	1·16	1·07	—	—
51	48 (2 ×)*	43	0·73	1·0	0·07	0·20
52	48 (2 ×)*	41	1·85	1·63	—	—
28	76	52	1·0	0·89	—	—

* Second injection of oestradiol at 24 hr.

creased permeability to glucose or oestrone oxidation of TPNH (Talalay & Williams-Ashman, 1958) or both may be involved.

Our attempts to find an effect of oestradiol added *in vitro* to any of the enzymes in the extracts investigated have had negative results, with either DPN or TPN as coenzyme. Hollander & Stephens (1959) were unable to show any effect of oestradiol in oxidizing DPNH by homogenates of rat uterus which were very active in oxidizing it when various phenols and chlorophenols were added in the presence of oxygen. This activity was found in particles which sedimented at 5000 *g* for 10 min. and 100 000 *g* for 1 hr. and none in the supernatant. Our extracts would contain some of the latter particles. The same authors found that homogenates of the uteri of ovariectomized rats did not oxidize DPNH in the presence of 2:4-dichlorophenol. Homogenates of uteri of ovariectomized animals which had received injections of 0·2 mg. of oestradiol 72 hr. previously had activities which were higher than the mean values from uteri of intact rats. The relationship of this enzyme to the enzymes we have studied has not been investigated.

The question of what happens to the increased phosphogluconate that is probably formed when glucose 6-phosphate dehydrogenase has increased and the phosphogluconate dehydrogenase has not, is not yet solved. Growth of *E. coli* in the presence of dinitrophenol is accompanied by a great increase in pyruvic acid in the medium and the extracts show increased glucose 6-phosphate dehydrogenase and no change in phosphogluconate dehydrogenase. This might indicate the operation of the Entner & Doudoroff (1952) pathway for splitting of oxidized phosphogluconate. However, we have been unable so far to find any degradation of phosphogluconate in the absence of TPN in extracts of the uterus.

Another parameter which may affect the activities of the TPN dehydrogenases is the presence of the TPNH oxidases, especially glutathione reductase. The analyses reported for reduced and total sulphydryl groups would indicate that insufficient glutathione was present to affect the measurement of dehydrogenase activities. There was insufficient material for an analysis of glutathione in the uteri of the present series. It may be during the period 2–8 hr. after oestradiol injection, when the dehydrogenases are low and there is increased protein in the tissue, that glutathione may be increased, especially the oxidized form. Glutathione analyses for another series will be reported later.

The time course of the response in enzyme activity may be compared with various other parameters of oestrogen-induced reaction of the ovariectomized rat uterus, as reported in the literature

Table 8. *Changes in the rat uterus after oestradiol*

Time (hr.)	Parameter	Change	Reference
1	Serum albumin ^{131}I	+	Kalman & Lowenstein (1958)
1	Uptake of O_2 (no glucose)	+	Szego & Roberts (1953)
2	Soluble protein	+	This paper
2	H_2O	+	Reynolds (1949)
2	^{22}Na	+	Kalman & Lowenstein (1958)
2	^{32}P	++	Kalman & Lowenstein (1958)
2	^{131}I	+	Kalman & Lowenstein (1958)
2	Glucose 6-phosphate and phosphogluconate dehydrogenase (endometrium)	−	This paper
4	Glucose uptake	+	Szego & Roberts (1953)
4	Lactate accumulation	+	Szego & Roberts (1953)
5	Incorporation of amino acids into protein	+	Mueller (1957)
6	Serine aldolase	+	Mueller (1957)
6	Incorporation of $^{14}CO_2$ into adenine	+	Mueller (1957)
6	Protein content	+	Telfer (1953)
6	Incorporation of acetate into lipid	+	Mueller (1957)
6	Ribonucleic acid content	−	Telfer (1953)
10	Uptake of O_2 (glucose)	+	Szego & Roberts (1953)
12	Glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase (endometrium)	+	This paper
12	Glycogen formation	+	Walaas (1952)
12	Uridine into ribonucleic acid	+	Mueller (1957)
13·5	Mitosis (mouse endometrium)	+	Allen, Smith & Gardner (1937)
15	Uptake of O_2 (glucose)	+	Szego & Roberts (1953)
16	Glucose 6-phosphate dehydrogenase (myometrium)	+	This paper
20	Lactate accumulation	−	Szego & Roberts (1953)
21	Deoxyribonucleic acid content	+	Telfer (1953)
21	Ribonucleic acid content	+	Telfer (1953)

(Table 8). These various experimental results, although performed in different laboratories with all the variability of procedure, animals and even dosage, do seem to fall into a logical sequence. The early changes suggest an effect of the oestrogens and other steroids on permeability, as has been noted before (Kalman & Lowenstein, 1958; Elliott & Yrarrazaval, 1952). There is evidence that the primary effect of oestradiol is on the capillaries, causing permeability to protein but not to red cells (Kalman, 1958; Zierler, 1958). We may note that the decrease in enzyme specific activity in the oestrogen-stimulated endometrium occurred at 2 hr., the time when Kalman showed that increased permeability of the capillaries allowed serum albumin to pass into the tissue and ^{22}Na and ^{131}I increased along with imbibition of water. Szego & Roberts (1953) found at 4 hr. after oestradiol injection a concomitant increase in glucose uptake and lactate accumulation, whereas the increase in oxygen uptake due to glucose occurred at 10 and 15 hr., at about the same times as the glucose 6-phosphate dehydrogenase started to increase. At 20 hr. glycolysis decreased. It would seem that the first reaction to increased glucose was glycolytic breakdown. It would be interesting to know whether this increased glycolysis was intracellular or extracellular or both (Shaw & Stadie, 1957), how much of the increased glucose uptake is accounted for by glycolysis and whether there was also an induced increase of glycolytic enzymes. It is to be noted that the increased glycolysis occurs before increased protein synthesis and decreases after it. The first synthetic enzymic activities to be increased are those at 5 hr., incorporating amino acids into protein (Mueller, 1957). An increase in protein content was noted at 6 hr. by Telfer (1953). Mueller found increased incorporation of acetate at the same time into lipid and carbon dioxide into adenine, and Telfer found a decreased ribonucleic acid: protein ratio. At 10 hr. oxygen uptake in the presence of glucose was increased (Szego & Roberts, 1953). At 12 hr. the dehydrogenases for glucose 6-phosphate and phosphogluconate started to show increased activity in the endometrium, glycogen was formed (Walaas, 1952) and uridine was incorporated into ribonucleic acid (Mueller, 1957). At 15 hr. the oxygen uptake again increased, and glucose 6-phosphate-dehydrogenase activity started to increase in the myometrium. At 20 hr. glycolysis was decreased. At 21 hr. the nucleic acid content was increased (Telfer, 1953). Allen, Smith & Gardner (1937) reported increased mitosis in the myometrium of the mouse 13·5 hr. after oestradiol but we have been unable to find comparable observations in the rat.

Thus we can see a logical sequence of metabolic events leading to, or accompanying, an increase in size of the uterus. However, the cause and effect of relationships are not necessarily evident from the

sequence. A study such as Beck's (1958) on the pacemakers of glycolysis in leucocytes would be desirable, as would be a more complete picture of the initial reactions on which Kalman (1958) has done such interesting work.

SUMMARY

1. The sequence of changes in the activities of glucose 6-phosphate and phosphogluconate dehydrogenases has been determined in extracts of the endometrium and myometrium of the uteri of ovariectomized rats, untreated and at selected times after treatment with oestradiol-17β.

2. The activity of the glucose 6-phosphate dehydrogenase in units/mg. of soluble protein was significantly less than that of controls from 2 to 10 hr. after oestradiol, but no difference was shown if the activity was calculated in terms of the insoluble residue of the uterus.

3. By both methods of calculation a significant increase in the activities of both enzymes of both extracts was found at later times up to 48 hr. and a return to control values at 76 hr.

4. The results of the determination of the specific activities of hexokinase of some of the extracts are reported.

5. Transhydrogenase was determinable in extracts of myometrium but not of endometrium.

6. Extracts of the adrenal glands of the same animals were analysed in the same way to serve as control tissues. The enzyme activities exhibited no changes correlated with oestradiol injection.

7. These data are discussed in relation to other changes reported in uteri of ovariectomized rats after injection of oestradiol.

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REFERENCES

Allen, E., Smith, G. M. & Gardner, W. U. (1937). *Anat. Rec.* **67**, Suppl. 3, 3.
Beck, W. S. (1958). *J. biol. Chem.* **232**, 251, 271.
Benesch, R. E., Lardy, H. A. & Benesch, R. (1955). *J. biol. Chem.* **216**, 663.
Bonting, S. L. & Jones, M. (1957). *Arch. Biochem. Biophys.* **66**, 340.
Cohen, S. S. (1951). *Nature, Lond.*, **168**, 746.
Dickens, F. & McIlwain, H. (1938). *Biochem. J.* **32**, 1615.
Elliott, K. A. C. & Yrarrazaval, S. (1952). *Nature, Lond.*, **169**, 416.
Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
Glock, G. E. & McLean, P. (1955*a*). *Biochem. J.* **61**, 381.
Glock, G. E. & McLean, P. (1955*b*). *Biochem. J.* **61**, 390.
Glock, G. E., McLean, P. & Whitehead, J. K. (1956). *Biochem. J.* **63**, 520.
Griffith, J. Q., jun. & Farris, E. J. (1942). *The Rat in Laboratory Investigations*. Philadelphia: J. B. Lippincott Co.
Hollander, V. P. & Stephens, M. L. (1959). *J. biol. Chem.* **234**, 1901.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Kalman, S. M. (1958). *J. Pharmacol.* **124**, 179.
Kalman, S. M. & Lowenstein, J. M. (1958). *J. Pharmacol.* **122**, 163.
Kaplan, N. O., Colowick, S. P. & Neufeld, E. F. (1953). *J. biol. Chem.* **205**, 1.
Kinoshita, J. H. (1957). *J. biol. Chem.* **228**, 247.
Mueller, G. C. (1957). *Cancer Res.* **17**, 490.
Reynolds, S. R. M. (1949). *Physiology of the Uterus*. New York: Paul B. Hoeber Inc.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies on Biosynthesis in Escherichia coli*. Washington, D.C.: Carnegie Institution of Washington Publ. 607.
Scott, D. B. M. (1956*a*). *Biochem. J.* **63**, 587.
Scott, D. B. M. (1956*b*). *Biochem. J.* **63**, 593.
Scott, D. B. M. & Chu, E. (1959). *Biochem. J.* **72**, 426.
Scott, D. B. M. & Cohen, S. S. (1953). *Biochem. J.* **55**, 33.
Scott, D. B. M., Rogers, M. L. & Rose, C. (1958). *J. Amer. chem. Soc.* **80**, 2165.
Seegmiller, J. E. & Horecker, B. L. (1951). *J. biol. Chem.* **192**, 175.
Shaw, W. N. & Stadie, W. C. (1957). *J. biol. Chem.* **227**, 115.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Spiro, M. J. & Ball, E. G. (1958). *J. biol. Chem.* **231**, 31.
Szego, C. M. & Roberts, S. (1953). *Recent Progr. Hormone Res.* **8**, 419.
Talalay, P. & Williams-Ashman, H. G. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 15.
Telfer, M. A. (1953). *Arch. Biochem. Biophys.* **44**, 111.
Tepperman, H. M. & Tepperman, J. (1958). *Diabetes*, **7**, 478.
Walaas, O. (1952). *Acta endocrinol., Copenhagen*, **10**, 175.
Weber, G. & Cantero, A. (1957). *Cancer Res.* **17**, 995.
Zierler, K. L. (1958). *Amer. J. Physiol.* **192**, 283.

Biochem. J. (1960) **77**, 64

The Isolation of *cis*-Δ^9-Heptadecenoic Acid from Butterfat

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Butterfat contains a wide range of fatty acids extending from C_2 to C_{26} (cf. Hilditch, 1956; Jack & Smith, 1956; Shorland & Hansen, 1957). Although the better-known constituents are the even-numbered saturated homologues from C_4 to C_{18}, and the even-numbered mono-unsaturated acids from C_{10} to C_{22}, it has been established in recent years that trace quantities of saturated branched-chain acids, saturated *normal* odd-numbered acids and saturated high-molecular-weight (C_{20} to C_{26}) components are also present. In addition, there is evidence for the occurrence in butterfat of C_{18} conjugated and unconjugated di- and poly-unsaturated acids, and C_{20} and C_{22} di- and poly-unsaturated non-conjugated acids (cf. Hilditch, 1956; Riemenschneider, 1957), as well as *trans* C_{16} and C_{18} acids (Bertram, 1928; Cornwell, Backderf, Wilson & Brown, 1953; Backderf & Brown, 1958).

In the present paper is reported the isolation from butterfat of an odd-numbered mono-unsaturated fatty acid: *cis*-Δ^9-heptadecenoic acid. Odd-numbered unsaturated fatty acids have not formerly been isolated from butterfat.

EXPERIMENTAL

The butterfat used in this investigation was prepared from fresh butter supplied by the Awahuri Dairy Co., Palmerston North, New Zealand, and was considered a typical sample of the butter made in early February 1959, with cream from predominantly Jersey-breed herds. The butterfat was prepared by repeatedly washing the melted butter in hot water, centrifuging and drying *in vacuo*.

Fatty acids were prepared by hydrolysing 3502·5 g. of butterfat by the conventional method recommended by Hilditch (1956). The fatty acids were steam-distilled and yielded 3099·0 g. of steam-non-volatile acids which in turn were separated into 'liquid' and 'solid' acids by crystallizing three times from acetone at −31°. For this investigation the 'liquids' were converted into methyl esters and 1469 g. was fractionally distilled *in vacuo* in a 460 cm. × 5 cm. glass vigreux column yielding 24 fractions (N2LL1–N2LL24) and a residue. One of these methyl ester fractions (N2LL14) with constants suggesting the presence of the methyl esters of C_{17} fatty acids (wt. 38·71 g.; m.p. −18·9 to −16·3°; saponification equiv. 288·8; iodine value 77·0) was denoted G1 and refractionated in a 50 cm. × 1·8 cm. column fitted with a coiled spring (column E, Shorland, 1952). Thirteen ester fractions (G1L1–G1L13) and a residue resulted, and the following, which were relevant to this investigation, were bulked as fatty acids and denoted G6: G1L3, 1·97 g., m.p. −7·5° to −2·5°, saponification equiv. 281·1; G1L4, 2·26 g., m.p. −9·5° to −8·6°, saponification equiv. 284·4; G1L5, 1·79 g., m.p. −7·7° to −6·9°, saponification equiv. 283·4; G1L6, 2·46 g., m.p. −7·2° to −6·3°, saponification equiv. 283·3; G1L7, 2·52 g., m.p. −7·2° to −6·3°, saponification equiv. 282·7; G1L8, 2·53 g., m.p. −13·5° to −12·0°, saponification equiv. 285·4.

Eighteen low-temperature crystallizations of G6 fatty acids from solvents, the final two being from 10 vol. of acetone and methanol respectively at −50°, yielded fraction G11S3S (wt. 1·42 g., m.p. 11·4–12·2°). Further purification of this fraction was effected by eluting the methyl esters through a silica-gel chromatographic column (wt. 20·0 g.) with 50 ml. charges of light petroleum containing 1% of ethyl ether. Of the 14 fractions obtained, the sixth to the eleventh inclusive, which had almost identical melting points, were combined and denoted G15 (wt. 0·80 g., m.p. −26·3 to −25·2°). Fraction G15, after conversion into fatty acids, possessed the following chemical and physical properties which identified it as *n*-heptadecenoic acid: m.p. 11·4–12·2° [reported values: 9–10° (Chisholm & Hopkins, 1957); 13·0–13·5° (Ames & Bowman, 1951) for synthetic *cis*-Δ^9-heptadecenoic acid; 14·5° (Appel, Böhm, Keil & Schiller, 1947)]; saponification equiv. 268·1 (Calc. for $C_{17}H_{32}O_2$: 268·4); combustion analysis: found: C 76·2; H 11·7. Calc. for $C_{17}H_{32}O_2$: C 76·1; H 12·0%); iodine value 83·1 (Calc. for *n*-heptadecenoic acid 94·6); methyl ester m.p. −26·3° to −25·2° (Appel *et al.* 1947, report −26·0°). Hydrogenation of G15 methyl esters in ethanol with Pt oxide as catalyst yielded a fraction which after purification by crystallization from 20 vol. of ethyl ether at −40° had m.p. 27·2–28·0° [Francis & Piper (1939) reported m.p. 29·7° for pure *n*-methyl heptadecanoate]. When the hydrogenated methyl ester (m.p. 27·2–28·0°) was mixed in equal proportions with authentic *n*-methyl heptadecanoate (m.p. 28·5–29·0°) it gave a mixed m.p. of 28·0–28·6°. The methyl ester when converted into fatty acid had m.p. 60·6–60·9° [Francis & Piper (1939) report m.p. 61·3° for pure *n*-heptadecanoic acid], and when mixed in equal proportions with pure *n*-heptadecanoic acid (m.p. 61·3–61·6°) gave a mixed m.p. of 60·7–60·9°

Gas–liquid-chromatographic analyses. With the original model of the Pye Argon Chromatograph, which incorporates the ionization detector introduced by Lovelock, James & Piper (1959), the methyl esters of fraction G15 were chromatographed first at 150° through a Celite column impregnated with 20% of polydiethylene glycol succinate and secondly at 200° through a Celite column containing 20% of Apiezon M as liquid phase. The mean of two R_F determinations with the polyester column was 3·68 (mean of five values found for authentic *n*-methyl heptadecenoate, R_F 3·67, S.D. ±0·06). With the Apiezon M column the R_F was 3·04.

Gas–liquid analysis of the hydrogenated methyl ester of fraction G15, with a Celite column containing 20% of polydiethylene glycol succinate at 150°, gave R_F 3·19 (mean of six values found for authentic *n*-methyl heptadecanoate, R_F 3·18, S.D. ±0·12). R_F values were determined relative to *n*-methyltetradecanoate.

Location of double bond. Oxidation of 0·17 g. of G15 methyl esters with $KMnO_4$ in acetic acid by the method described by Haverkamp Begemann, Keppler & Boekenoogen (1950) yielded 0·08 g. of monobasic acids soluble in light petroleum and 0·06 g. (m.p. 101–103°) of dibasic acids insoluble in light petroleum. The weight of unoxidized neutral material soluble in ethyl ether after washing with Na_2CO_3 was 0·11 g. The dibasic acids were crystallized three times from water and yielded 0·03 g. of azelaic acid, m.p. 104·0–105·2°, and gave a mixed m.p. of 104·5–105·7° with equal proportions of pure azelaic acid (m.p. 105·1–105·7°). The acids soluble in light petroleum when subjected to gas–liquid-chromatographic analysis in the apparatus described by James & Martin (1952) were shown to contain the following constituents (expressed in moles %): *n*-hexanoic (?) acid 2·7%; *n*-heptanoic (?) acid 8·0%; *n*-octanoic acid 81·3%; *n*-nonanoic acid 8·0%. In order to determine the weight of *n*-octanoic acid in the total acids soluble in light petroleum, 0·0726 g. of acids was made up to 5 ml. in ethyl ether and a sample (0·25 ml.) was applied to the gas–liquid chromatogram by means of a hypodermic syringe. The analysis showed that 0·001921 g. of *n*-octanoic acid and 0·0001985 g. of *n*-nonanoic acid were detected from the 0·00363 g. applied, indicating that 58·9% by weight of the charge passed through the column and was detected.

Infrared analysis carried out with a Perkin–Elmer model 21 spectrophotometer showed fraction G15 to contain no *trans* acids.

Fraction G11S3S(*l*). A further fraction G11S3S(*l*) obtained as the twelfth fraction after chromatographing through silica gel, and referred to earlier, appears to be purer than G15. This methyl ester fraction (wt. 0·08 g.), m.p. −23·5° to −22·2°, on gas–liquid-chromatographic analysis with the polyester column was shown to contain 96·4 moles % of *n*-methylheptadecenoate, 1·4 moles % of 14- or 15-methylhexadecanoate and 2·2 moles % of *n*-methyloctadecanoate.

Combustion analysis and melting points. Combustion analyses were carried out by Dr A. D. Campbell, Chemistry Department, University of Otago, New Zealand. Melting points were determined in closed capillaries and are uncorrected.

DISCUSSION

The chemical and physical properties of fraction G15 isolated in this work identify it as the straight-chain C_{17} unsaturated fatty acid *cis*-Δ^9-heptadecenoic acid. Its *cis* configuration is established by infrared analysis, which revealed an absence of *trans* acids. The position of the double bond was determined by oxidation of 0·17 g. of sample G15 with $KMnO_4$ in acetic acid and subsequent gas–liquid-chromatographic analysis (James & Martin, 1952) of the resulting 0·08 g. of monobasic fatty acids soluble in light petroleum. It was found that 58·9% by weight of a sample of the acids soluble in light petroleum was detected by gas–liquid chromatography and *n*-octanoic acid constituted 90·6% by weight of these detected volatile acids. Based on the total weight (0·08 g.) of acids soluble in light petroleum produced in the oxidation, 58·9% represents 0·047 g. of volatile fatty acids including 0·043 g. of *n*-octanoic acid. Gas–liquid-chromatographic analysis of the methyl esters of fraction G15 showed it to contain only 90·4% of *n*-heptadecenoic acid, there being present as impurities 6·2 moles % of 14- or 15-methylhexadecanoic acid and 3·4 moles % of *n*-octadecenoic acid. After allowing for this 9·6% of impurities, the calculated weight of *n*-octanoic acid produced on oxidation of the original 0·17 g. of the fraction is 0·079 g. and the weight of *n*-octanoic acid found (0·043 g.) represents approximately 54% of the calculated yield. This establishes most of fraction G15 as having the double bond in the 9:10 position as is characteristic of animal fats.

Further confirmation of the location of the double bond in fraction G15 is provided by the yield of azelaic acid on oxidation with potassium permanganate in acetic acid.

Although odd-numbered unsaturated *normal* fatty acids have not formerly been isolated from butterfat, a straight-chain C_{17} mono-unsaturated fatty acid (*n*-heptadecenoic acid) has been found in human-hair fat (Weitkamp, Smiljanic & Rothman, 1947), lamb-caul fat (Shorland & Jessop, 1955), musk-ox body fat (Chisholm & Hopkins, 1957), shark-liver oil (I. M. Morice & F. B. Shorland, unpublished work) and sheep perinephric fat (R. P. Hansen, F. B. Shorland & N. J. Cooke, unpublished work). Weitkamp *et al.* (1947) provided evidence to show that the trace amounts of C_{17} unsaturated acids present in human-hair fat yielded *n*-undecanoic and *n*-nonanoic acids on oxidation with potassium permanganate in acetone and comprised a mixture of 80% of Δ^8 and 20% of Δ^8 isomers of *n*-heptadecenoic acid.

Based on the weight of pure *n*-heptadecenoic acid isolated in this work, together with the amounts estimated to be present in accompanying impure fractions, the content of this fatty acid in the sample of butterfat investigated is estimated to be 0·06% of the total weight of fatty acids. Gas–liquid-chromatographic analysis, however, suggests the total content of *n*-heptadecenoic acid in this sample to be approximately 0·2% of the total weight of fatty acids. In the body fat of musk-ox the amount of *n*-heptadecenoic acid present was estimated by Chisholm & Hopkins (1957) to be 0·9% of the weight of fatty acids.

The acknowledged complexity of the fatty acid composition of butterfat is further illustrated by the present isolation of *cis*-Δ^9-heptadecenoic acid, which represents the fourth C_{17} fatty acid to be isolated from butterfat, the others being *n*-hepta-

decanoic (margaric) acid (Hansen, Shorland & Cooke, 1957) and the branched-chain (+)-14-methylhexadecanoic acid and 15-methylhexadecanoic acid (Hansen & Shorland, 1951).

SUMMARY

1. *cis*-Δ^9-Heptadecenoic acid has been isolated from butterfat and conclusively identified.

2. The weight of *cis*-Δ^9-heptadecenoic acid isolated from the sample of butterfat under investigation represented approximately 0·06% of the total weight of fatty acids.

3. Odd-numbered unsaturated fatty acids have not formerly been found in butterfat.

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REFERENCES

Ames, D. E. & Bowman, R. E. (1951). *J. chem. Soc.* p. 1079.
Appel, H., Böhm, H., Keil, W. & Schiller, G. (1947). *Hoppe-Seyl. Z.* **282**, 220.
Backderf, R. H. & Brown, J. B. (1958). *Arch. Biochem. Biophys.* **76**, 15.
Bertram, S. H. (1928). *Biochem. Z.* **197**, 433.
Chisholm, M. J. & Hopkins, C. Y. (1957). *Canad. J. Chem.* **35**, 1434.
Cornwell, D. G., Backderf, R. H., Wilson, C. L. & Brown, J. B. (1953). *Arch. Biochem. Biophys.* **46**, 364.
Francis, F. & Piper, S. H. (1939). *J. Amer. chem. Soc.* **61**, 1939.
Hansen, R. P. & Shorland, F. B. (1951). *Biochem. J.* **50**, 207.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1957). *Nature, Lond.*, **179**, 98.
Haverkamp Begemann, P., Keppler, J. G. & Boekenoogen, H. A. (1950). *Rec. Trav. Chim. Pays-Bas*, **69**, 439.
Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed. London: Chapman and Hall Ltd.
Jack, E. L. & Smith, L. M. (1956). *J. Dairy Sci.* **39**, 1.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Lovelock, J. E., James, A. T. & Piper, E. A. (1959). *Ann. N.Y. Acad. Sci.* **72**, 720.
Riemenschneider, R. W. (1957). *Proc. Conf. Milk Concentrates; East. Util. Res. Develop. Div. agric. res. Serv., U.S. Dept. Agric.*
Shorland, F. B. (1952). *J. appl. Chem.* **2**, 438.
Shorland, F. B. & Hansen, R. P. (1957). *Dairy Sci. Abstr.* **19**, 168.
Shorland, F. B. & Jessop, A. S. (1955). *Nature, Lond.*, **176**, 737.
Weitkamp, A. W., Smiljanic, A. M. & Rothman, S. (1947). *J. Amer. chem. Soc.* **69**, 1936.

Biochem. J. (1960) **77**, 66

The Effects of some Ions and Chelating Agents on the Oxidase Activity of Caeruloplasmin

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Caeruloplasmin, the blue cuproprotein of plasma, has oxidase activity against a variety of substrates. Holmberg & Laurell (1951) showed that the activity was markedly affected by anions. Complex effects were obtained, activity being increased by anions in low concentration and decreased by the same anions in higher concentration. Broman (1958) reported inhibition of crude caeruloplasmin by dicarboxylic acids and ethylenediaminetetraacetic acid. It was suggested that the effect of the latter was due not to a direct interaction with caeruloplasmin, but to the removal of catalytically active metal–protein complexes present as contaminants.

In the work described below, the effects of some anions, cations and chelating agents on the oxidase activity of purified caeruloplasmin were investigated.

METHODS AND MATERIALS

The apparatus was washed and the solutions were made up as described by Curzon & Vallet (1960).

Chemicals. A.R. grades were used whenever obtainable. *NN*-Dimethyl-*p*-phenylenediamine dihydrochloride (British Drug Houses Ltd., Poole, Dorset, England), supplied in sealed ampoules, was subdivided into 100 mg. lots and resealed into ampoules under vacuum and stored at −25°. Purity was 99% with respect to free base. Trace-metal contamination was 10 p.p.m. of Ba, 4 p.p.m. of Al, Ca, Fe, Mg, Na and Ni, and 0·4 p.p.m. of Cr and Mn. These figures are rough estimates obtained from the estimated

upper limits by spectrographic analysis and the sulphated ash content provided by the manufacturers. Tests for Fe were also carried out with $\alpha\alpha'$-dipyridyl (Vogel, 1946) with ammonium sulphide to reduce ferric iron. A negative reaction was obtained. The lowest concentration of Fe detectable by this method in the presence of substrate was equivalent to 10 p.p.m. of substrate. Solutions of substrate were stored at $-25°$.

Measurement of pH. A pH meter with an accuracy of $\pm 0{\cdot}02$ pH unit was used. Usually no change in pH was detectable during determinations of oxidase activity, though occasionally changes of $> 0{\cdot}03$ pH unit were observed.

Caeruloplasmin. This was prepared from the G2 fraction of human plasma by the method of Curzon & Vallet (1960), which includes a final purification on a diethylaminoethyl-cellulose column. Selected eluate fractions were dialysed against water at 4° and stored at 4°. Oxidase activity of the caeruloplasmin so obtained was 44–48 units/ml. A unit was defined as the activity of an amount of caeruloplasmin which on incubation with *NN*-dimethyl-*p*-phenylenediamine oxalate, under conditions described by Curzon & Vallet (1960), resulted in an $E_{1\,cm.}$ at 550 mμ of 0·10 (21 units ≡ 1 μg. of caeruloplasmin copper). Activity of the preparations fell 2–4% during 4–6 weeks of storage at 4°. Curzon & Vallet (1960) found that comparable preparations lost activity more rapidly at $-25°$. The reason for this is not known.

Determination of caeruloplasmin activity. NN-Dimethyl-*p*-phenylenediamine was oxidized by caeruloplasmin and the extinction (E) of the red reaction product measured at 550 mμ.

The method described by Curzon & Vallet (1960) was worked out specifically for following caeruloplasmin activity through the stages of its purification from plasma. However, this method involves a too complex ionic environment to be of use in the study of the effects of ions on activity, for both acetate buffer and sodium chloride are present. Moreover, the substrate was used as the oxalate salt. This salt is more stable in the solid state and thus has certain advantages over the more frequently used hydrochloride. Also the oxalate ion inhibits non-enzymic oxidation of the substrate by contaminant metal. A serious disadvantage, however, in kinetic work is that oxalate causes significant inhibition of enzyme activity, resulting in an obscuring of the effects of other inhibitors and in a deviation from the Michaelis curve at high substrate concentation. Therefore *NN*-dimethyl-*p*-phenylenediamine dihydrochloride was used. With this substrate, results fitted Michaelis curves, and linear Lineweaver–Burk graphs were obtained.

To 2·0 ml. of acetate buffer, pH 5·5, of chosen molarity were added 1·0 ml. of either water or a solution of the substance under investigation and 1·0 ml. of substrate solution. After equilibration for 5 min. in a water bath at 25° or 37°, 1·0 ml. of caeruloplasmin solution at the same temperature was added. Appropriate blanks were run simultaneously. After 30 min. at 25° or 15 min. at 37° the reaction was stopped by adding 2·0 ml. of 0·3 mM-azide and $E_{1\,cm.}$ was read at 550 mμ as described by Curzon & Vallet (1960), the only difference being that when determinations were performed at 25°, the azide was added at room temperature. It was found convenient to run 15–20 tubes at a time and to read extinctions within 7 ± 2 min. of adding the azide so that errors, due to any slow changes in extinction, were negligible. All determinations were duplicated and variation of duplicates from the mean averaged ±1%.

RESULTS

Preliminary experiments. When caeruloplasmin was incubated with substrate at 25° or 37° as described above, $E_{550\,m\mu}$ was directly proportional to the amount of caeruloplasmin within the range, $E_{1\,cm.}$ 0·10–0·60. Caeruloplasmin concentrations in subsequent experiments were chosen so that this range was not exceeded. With incubation for 15 min. at 37°, $E_{1\,cm.} = 2{\cdot}3 \times$ units of caeruloplasmin $= 48 \times$ μg. of caeruloplasmin copper. $E_{1\,cm.}$ was also directly proportional to the time of incubation with substrate. In the presence of 0·03 M-sodium chloride or 0·2 mM-sodium oxalate inhibition occurred, but a linear relationship of $E_{1\,cm.}$ with time was still maintained. Thus $E_{1\,cm.}$ after 15 min. incubation at 37° or 30 min. incubation at 25° may be used as a measure of initial velocity of the enzymic oxidation. There was no evidence of any

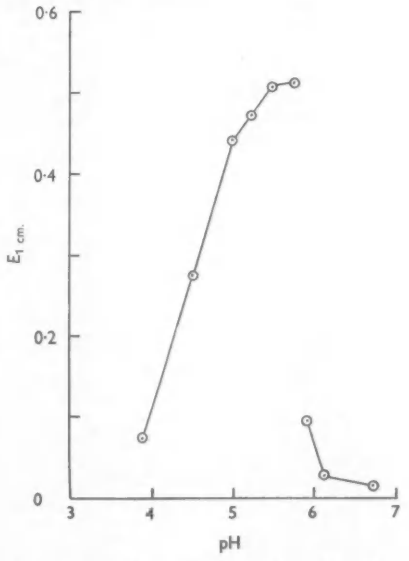

Fig. 1. Effect of pH on caeruloplasmin activity. Buffers were 0·08 M. Acetate buffers were used between pH 4·0 and 5·75 and phosphate buffers between pH 5·68 and 6·7. Incubation mixtures contained substrate, 0·34 mM-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride; caeruloplasmin, 2·6 units by the method of Curzon & Vallet (1960), in a total volume of 5 ml. Incubation was at 37° for 15 min. The reaction was stopped by addition of 2·0 ml. of 0·3 mM-sodium azide and $E_{1\,cm.}$ at 550 mμ was measured.

induction period. All kinetic investigations were done at pH 5·5, which appears to be optimum for caeruloplasmin activity under the present experimental conditions (Fig. 1). There are difficulties in the determination of pH–activity curves, as acetate buffers have sufficient buffering capacity only in the range pH 4·0–5·8, and above pH 5·8, effective buffers are themselves relatively inhibitory. It is apparent from Fig. 1 that phosphate is strongly inhibitory relative to acetate. The pH–activity curve with *NN*-dimethyl-*p*-phenylenediamine as substrate is similar to that found by Holmberg & Laurell (1951), who used *p*-phenylenediamine.

Comparison of the inhibitory effects of some anions. The anions, thiocyanate, fluoride, chloride, bromide and sulphate (in decreasing order of effect) inhibit the oxidase activity of caeruloplasmin. Plots of $1/E$ against salt concentration are reasonably linear (Fig. 2). The effect of chloride was investigated more fully. The degree of inhibition was the same whether lithium, sodium or potassium chloride was used; it was not influenced by pretreatment of the caeruloplasmin with chloride, inhibition being dependent only on the chloride concentration present during incubation with substrate.

Acetate buffer also has some inhibitory effect but in this case the graph of $1/E$ against acetate concentration is concave (Fig. 5).

Kinetics of inhibition

Acetate buffer. The effect of varying the concentration of acetate buffer, pH 5·5, on activity at various substrate concentrations was determined and expressed in the Lineweaver–Burk form. A series of lines was obtained with a definite suggestion of parallelism (Fig. 3). It was only possible to produce limited activity changes by varying acetate concentration and thus there was some doubt about the significance of this. Parallel Lineweaver–Burk graphs indicate an anti-competitive mechanism in which the inhibitor combines with the enzyme–substrate complex but not with the free enzyme (Burk, 1944).

Chloride. When the buffer concentration was kept constant and inhibition by chloride determined at various substrate concentrations, Lineweaver–Burk graphs were clearly parallel (Fig. 4). Thus chloride is an anti-competitive inhibitor. The general equation for anti-competitive inhibition,

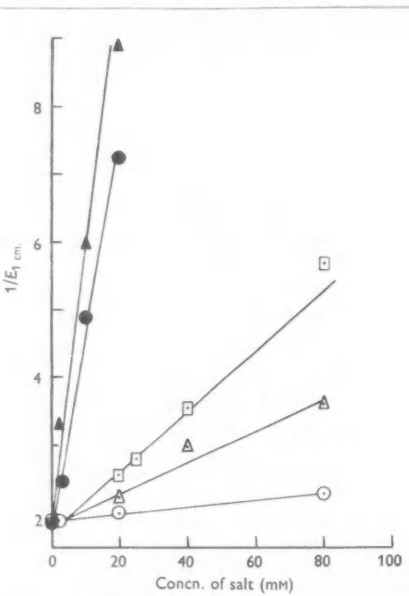

Fig. 2. Effect of anions on caeruloplasmin activity. The buffer was 0·08 M-acetate, pH 5·5; other conditions were as in Fig. 1. ⊙, SO_4^{2-}; △, Br^-; □, Cl^-; ●, F^-; ▲, SCN^-. Sodium salts were used, except for SCN^-, which was the potassium salt; the change of cation had no effect (see text).

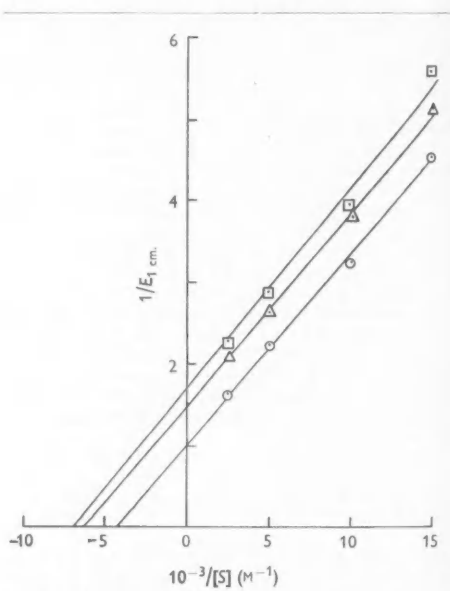

Fig. 3. Effect of variation of the concentration of acetate buffer, pH 5·5, on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver–Burk graph; general conditions were as in Fig. 1 but the caeruloplasmin present was 2·9 units. ⊙, 0·01 M-Acetate; △, 0·2 M-acetate; □, 0·4 M-acetate.

assuming Michaelis kinetics (Friedenwald & Maengwyn-Davies, 1954), is:

$$1/V = 1/V_{max.} + K_s/V_{max.}S + I/V_{max.}K_i,$$

where V is the reaction velocity at substrate concentration S; $V_{max.}$, the maximum velocity in the presence of inhibitor; K_s, the dissociation constant of the enzyme–substrate complex; K_i, the dissociation constant of enzyme–substrate–inhibitor complex to enzyme–substrate complex + inhibitor; I, the concentration of inhibitor

From this it may be shown that the negative intercepts on the $1/S$ axis (the reciprocals of the apparent Michaelis constants) are directly proportional to inhibitor concentration:

$$1/K_m = 1/K_m^0(1 + I/K_i),$$

where K_m is the Michaelis constant, at inhibitor concentration I and K_m^0, the Michaelis constant at zero concentration of the inhibitor.

The intercept and gradient of the $(1/K_m)$–I graph (not shown) give $1/K_m^0$ and $1/(K_m^0K_i)$ respectively. Thus K_i can be evaluated graphically and for chloride a value of 28 mM was obtained. In acetate inhibition the curve of $1/E$ against acetate concentration is concave, there is thus no linear relationship between $1/K_m$ and acetate concentration, and K_i is not directly obtainable.

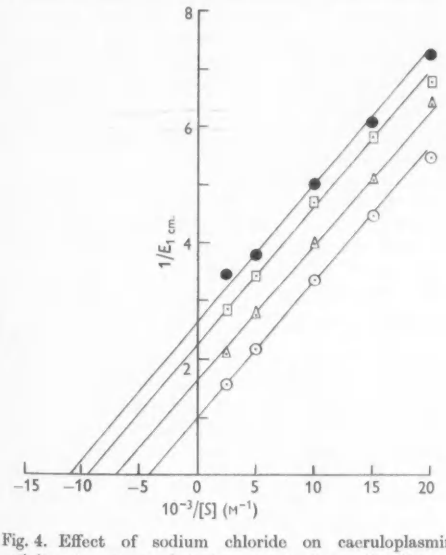

Fig. 4. Effect of sodium chloride on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver–Burk graph. General conditions were as in Fig. 2. ⊙, Sodium chloride absent; △, 0·02 M-sodium chloride; ⊡, 0·04 M-sodium chloride; ●, 0·05 M-sodium chloride. The caeruloplasmin present was 2·9 units.

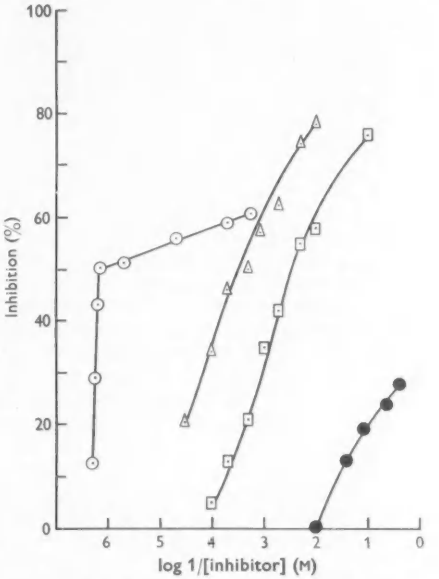

Fig. 5. Effect of carboxylic acids on caeruloplasmin activity. Conditions were as in Fig. 2, except that the mixture was incubated at 25° for 30 min. and 3·3 units of caeruloplasmin were present. The acids were in the form of sodium salts. The effect of variation of concentration of acetate buffer, pH 5·5, is also shown, zero inhibition referring to activity in 0·01 M-buffer. Conditions were as in Fig. 2 except that the caeruloplasmin present was 2·25 units. ⊙, EDTA; △, oxalic acid; ⊡, malonic acid; ●, acetate buffer, pH 5·5.

Table 1. *Effect of some carboxylic acids and chelating agents on caeruloplasmin activity*

The reaction mixture contained 0·08 M-acetate, buffer, pH 5·5, 0·34 mM-*NN*-dimethyl-*p*-phenylenediamine dihydrochloride, 3·3 units of caeruloplasmin in a total volume of 5 ml. Incubation was at 25° for 30 min. The reaction was stopped by 2·0 ml. of 0·3 mM-sodium azide and $E_{1\ cm.}$ read at 550 mμ. The carboxylic acids and diamines were used as the disodium salts and the sulphates respectively.

Inhibitor	Concn. (mM)	Inhibition (%)
Succinic acid	2	0
Glutaric acid	2	−2
Maleic acid	0·05	34
Maleic acid	0·01	23
Fumaric acid	0·2	0
Phthalic acid	10	12
*iso*Phthalic acid	10	−3
Terephthalic acid	10	4
1:2-Diaminoethane	10	20
1:3-Diaminopropane	10	11
1:4-Diaminobutane	10	6

Chelating agents. The inhibitory effects of acetic acid, oxalic acid, malonic acid and ethylenediaminetetra-acetic acid (EDTA) are shown in Fig. 5 and the results of tests on some related substances are summarized in Table 1. Oxalic acid was a more powerful inhibitor than malonic acid and higher saturated aliphatic dicarboxylic acids had no effect. Maleic acid was an inhibitor but fumaric acid at comparable concentration was without effect. The phthalic acids had insignificant effects whereas 1:2-diaminoethane, which is a powerful chelator, and its higher homologues were only weakly inhibitory. The effect of EDTA is of some interest as there is a sharp discontinuity in the graph of inhibition against log (inhibitor concentration). This has been observed with crude preparations by Broman (1958), who attributed it as probably due to part of the oxidase activity being associated with loose protein–metal complexes, which are present as impurities but from which the metal is removable by EDTA. This is unlikely in the present experiment as purified caeruloplasmin was used. However it has been shown (see below) that trace metals, particularly iron, have an apparent activating effect on caeruloplasmin. The possibility of significant activating metal being

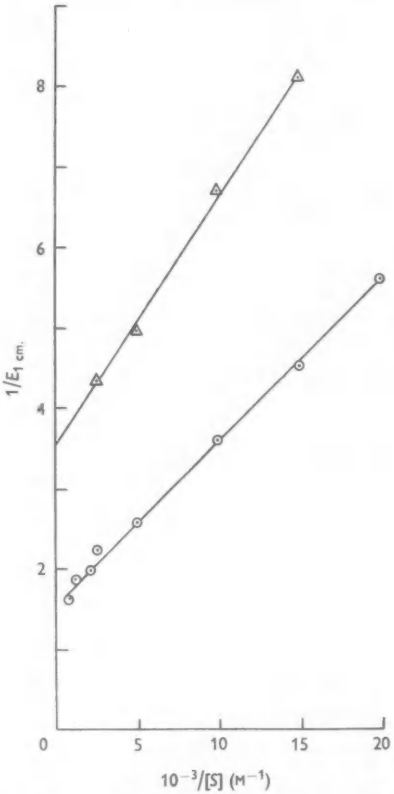

Fig. 6. Effect of EDTA on caeruloplasmin activity at various substrate concentrations. Results are plotted as a Lineweaver–Burk graph. General conditions were as in Fig. 5; caeruloplasmin present was 3·1 units. ⊙, EDTA absent; △, 1 μM-EDTA.

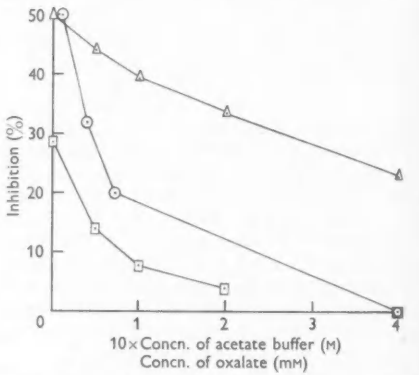

Fig. 7. Protection by acetate buffer and sodium oxalate of caeruloplasmin activity against inhibition by sodium chloride. General conditions were as in Fig. 1. ⊙, 0·02 M-Sodium chloride in the presence of acetate buffer; caeruloplasmin, 2·2 units; △, 0·02 M-sodium chloride in the presence of sodium oxalate; ⊡, 0·01 M-sodium chloride in the presence of sodium oxalate. The sodium oxalate experiments were all done in 0·01 M-acetate buffer with 2·9 units of caeruloplasmin.

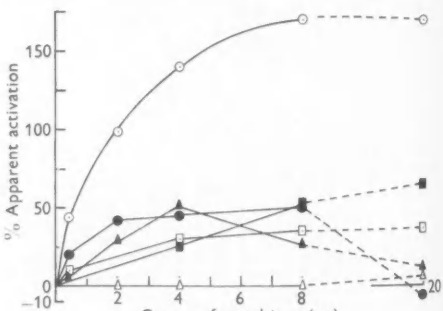

Fig. 8. Apparent activation of caeruloplasmin by metals. Conditions were as in Fig. 5. ⊙, Fe^{2+}; △, Mn^{2+}; ⊡, Co^{2+}; ●, Ni^{2+}; ▲, Zn^{2+}; ■, Fe^{3+}. The metals were used as sulphates except for Ni^{2+} and Fe^{3+} which were the nitrate and chloride respectively. The caeruloplasmin present was 1·4 units.

present as a contaminant in the enzyme, substrate or buffer was therefore investigated; particular attention was paid to iron. Caeruloplasmin preparations were tested for iron with $\alpha\alpha'$-dipyridyl as described above, and gave no detectable colour. This means that in the mixture for determination of activity, iron from the caeruloplasmin was $< 10^{-7}$ M. The linear relationship between activity and caeruloplasmin concentration is evidence against activating metal being present in the enzyme preparation unless so much is present that the enzyme is saturated with it (Dixon & Webb, 1958). Also, the addition of enzyme that had been inactivated by acid and subsequently neutralized, in equivalent amount to the intact caeruloplasmin, had no effect on the activity. This is evidence against activating metals being present as a contaminant in the caeruloplasmin.

There was also no evidence of significant activating metal being present in the substrate. Neither purification by vacuum sublimation nor a tenfold increase of the trace metals known to be present had significant effect on caeruloplasmin activity or its inhibition by EDTA. In addition the linear Lineweaver–Burk graphs obtained even at high substrate concentrations are evidence against the presence of activating metal in the substrate.

Finally, activating metal was not detectable in the buffer. Redistillation of the acetic acid and removal of trace metals from the sodium acetate by dithizone and 8-hydroxyquinoline extraction had no significant effect on caeruloplasmin activity or EDTA inhibition under conditions as in Fig. 5. Thus in general it appeared reasonable that the partial inhibition of caeruloplasmin by EDTA was due not to removal of 'complexable' activating metal but to a direct effect of EDTA on the caeruloplasmin (see Discussion).

The effect of substrate concentration on the inhibition of caeruloplasmin by EDTA is shown in Fig. 6. The inhibition was non-competitive and the partially inhibited enzyme lowered the Michaelis constant.

Protection of caeruloplasmin against chloride inhibition by acetate or oxalate. Inhibition of oxidase activity by chloride ions decreased as the concentration of the acetate buffer increased (Fig. 7). Similarly, at a fixed concentration of acetate buffer the inhibitory effect of chloride decreased in the presence of oxalate. Thus both acetate and oxalate protect caeruloplasmin against inhibition by chloride.

Effect of heavy metals

As it has been suggested that inhibition by EDTA was due to removal of metal ions, the effects on activity of various metallic salts was investigated.

Inhibition. The inhibitory effects of some heavy-metal ions are shown in Table 2. Fe^{2+} ions caused considerably increased activity in the first few minutes of incubation followed by inhibition. Higher concentrations of Fe^{2+} ions, but not of other metal ions tested, caused fading of the red oxidation product and Fe^{3+} itself oxidized the substrate. Thus in addition to enzymic effects the following equilibrium reaction may occur:

$$NN\text{-dimethyl-}p\text{-phenylenediamine} + Fe^{3+} \rightleftharpoons \text{Red oxidation product} + Fe^{2+}.$$

It was not convenient to study the effect of adding copper at concentrations comparable to those of related metals because of the direct catalytic effect of Cu^{2+} on the oxidation of the substrate. Ni^{2+} ions, Hg^{2+} ions and the tervalent ions Al^{3+} and In^{3+} showed considerable inhibitory power. The effect of In^{3+} ions was particularly marked, 2 μM-In^{3+} causing a 50% inhibition at a caeruloplasmin-copper concentration of 0·6 μM.

Activation. Activation by low concentrations of Fe^{2+} ions (see above) was confirmed and compared with the effects of other metals (Fig. 8). Comparison is made difficult by the inhibitory effects, which become obvious at higher concentrations. However, at 2 μM-metal the order of activating power of the ions is $Fe^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$. Fe^{3+} ions caused less activation than Fe^{2+} ions. 2 μM-Ca^{2+}, -Mg^{2+}, -Mn^{2+} and -Cu^{2+} ions gave no activation and 2 μM-Cr^{3+} ions inhibited slightly. Cu^{2+} and Fe^{3+} ions were the only metal ions tested which caused any direct oxidation of the substrate.

DISCUSSION

Holmberg & Laurell (1951) showed that low concentrations of anions activated caeruloplasmin, whereas higher concentrations caused inhibition. In the present study, anion inhibition only was found, presumably because the acetate buffer present was sufficient to saturate the relevant

Table 2. *Inhibition of caeruloplasmin activity by metal ions*

General conditions were as given in Table 1. The metals were used as sulphates with the exceptions of Hg^{2+} and Fe^{3+}, which were chlorides, and Ni^{2+}, which was used as the nitrate. Concentrations marked with an asterisk are approximate.

Metal ions	Concn. (μM)	Inhibition (%)
Fe^{2+}	200	43
Co^{2+}	200	−4
Ni^{2+}	200	57
Zn^{2+}	200	30
Hg^{2+}	10*	50
Fe^{3+}	200	23
Al^{3+}	25*	50
In^{3+}	2·5*	50

activation centres. Ions may cause enzyme inhibition by a non-specific suppression of ionization of the centres in the enzyme which are necessary for activity. With this mechanism, an example of which is the inhibition of urease by salts (Kistiakowsky & Shaw, 1953), inhibition is entirely related to ionic strength and is independent of the nature of the ions present. It is apparent from the differences in inhibitory power shown by the ions investigated here that this non-specific effect is not a major factor in the inhibition of caeruloplasmin activity by anions. The order of inhibitory power of the anions thiocyanate > fluoride > chloride > bromide > sulphate is in agreement with Holmberg & Laurell's (1951) finding thiocyanate > chloride > nitrate.

Caeruloplasmin partly inhibited by acetate and oxalate is protected against chloride inhibition. This indicates that these ions bind either at the same inhibitory sites as chloride ions or at sites so close that binding of chloride ions at one group of sites is prevented by the presence of acetate or oxalate at adjacent sites. A practical conclusion that can be drawn is that in the determination of plasma-caeruloplasmin activity it is advantageous to use a concentrated acetate buffer. Although this results in decreased sensitivity, it also decreases interference by anions.

Chloride, and probably acetate, apparently inhibit caeruloplasmin by the anti-competitive or uncompetitive mechanism (Burk, 1944; Friedenwald & Maengwyn-Davies, 1954) in which the inhibitor combines with the enzyme–substrate complex but not with the free enzyme. Few examples of anti-competitive inhibition are known. Dodgson, Spencer & Williams (1956) showed that inhibition of the arylsulphatase of *Alcaligenes metalcaligenes* by cyanide and hydrazine was an example and suggested that the inhibition of pepsin by hydrazine (Schales, Suthon, Roux, Lloyd & Schales, 1948) was explicable by the same mechanism. Lineweaver–Burk graphs of the inhibition of fumarase by sodium chloride (Alberty & Bock, 1953) suggest that this may be a further example. It is not known if the inhibitory anions are bound to the caeruloplasmin–substrate complex in the region of the point of substrate attachment. However, this is a reasonable assumption since modification of the enzyme by the substrate is most likely in this area. Anti-competitive inhibition by anions implies either that the enzyme–substrate complex possesses a site which is not present in the free enzyme and to which the inhibitor can attach itself (e.g. the arylsulphatase–*p*-nitrophenyl sulphate complex studied by Dodgson, Spencer & Williams, 1955) or that substrate binding induces a change in enzyme geometry so that sites become available for anion binding.

Chloride and acetate ions could possibly inhibit activity by forming caeruloplasmin–copper–anion complexes, substituting singly in the co-ordination shell of copper. Mapson (1941) found catalysis of ascorbic acid oxidation by copper ions to be inhibited by chloride and suggested that this was due to combination of copper with chloride ions. The availability of the copper of the enzyme–substrate complex but not of the resting enzyme, to certain ligands such as chloride ions, finds a parallel in the work of Scheinberg & Morell (1957), who showed that caeruloplasmin copper is available for exchange with ^{64}Cu only in the presence of substrate.

The relative inhibitory effects of the saturated dicarboxylic acids is, to some extent, explicable by the relative stabilities of the copper complexes. Peacock & James (1951) showed that as the number of carbon atoms between the carboxyl groups increased, the complex stability decreased. The reasons why maleic acid and oxalic acid are of comparable inhibitory power and the reasons for the lack of effect of fumaric acid and phthalic acid and ethylenediamine are not clear. Vallee (1955) points out that stabilities of complexes obtained with simple ionic systems are not necessarily related to the more complex metal–protein–inhibitor complexes. The relative inhibitory effects of carboxylic acids found here are considerably different from the order maleic acid > fumaric acid > salicylic acid, phthalic acid > oxalic acid, found by Broman (1958) in the presence of EDTA. This is probably due to differences in pretreatment of the caeruloplasmin and of pH, buffer and incubation temperature. Scheinberg, Aisen & Morell (1959) obtained evidence from titration curves that copper–carboxyl bonds are present in caeruloplasmin. Thus inhibition by carboxylic acids may involve breaking of these bonds and formation of new bonds with the inhibitor. As the copper–carboxyl bond is largely ionic (Sen, Mizushima, Curran & Quagliano, 1955), this would probably require little activation energy.

Low concentrations of metal ions, in particular Fe^{2+}, apparently increase caeruloplasmin activity. Therefore the discontinuous graph of inhibition against log [EDTA] and the concave $1/v$–inhibitor-concentration graphs for oxalate and malonate could well have been due to removal of contaminant activatory metal. Effects of EDTA on activities of non-metallic enzymes are usually explained as being due to chelation of contaminant metal. Interpretation is obviously more difficult when the enzyme itself contains metal. Considerable evidence was obtained mainly from kinetic measurements, and from the lack of effect of purification procedures on inhibition by EDTA, against significant contaminant activating metal being present in the caeruloplasmin, buffer and

substrate used. However, the findings that 0·01 mg. of purified iron-binding β-globulin/ml. inhibits activity 40%, and that 2 mg. of iron-binding β-globulin/ml. only inhibits to a slightly greater extent, strongly indicate that iron is present at very approximately 10^{-7} M in the system (G. Curzon, added 13 July 1960) as the iron-binding capacity of the β-globulin was about 0·5 mg. of Fe/g. of protein at pH 5·5. Thus the inhibition by EDTA is very possibly largely due to chelation of trace amounts of iron. The sharp break in the curve of inhibition against log [EDTA] was not found when the oxalate salt of the substrate was used (Curzon & Vallet, 1960). Under these conditions it is obscured by the chelating effect of oxalate on trace iron.

Activation by metal ions at low concentration with inhibition at higher concentration has frequently been shown in enzyme studies (Dixon & Webb, 1958). Caeruloplasmin appears particularly susceptible to metal ions, both activation and inhibition occurring at very low concentration. The marked activatory effect of ferrous ions is explicable by a coupled iron–caeruloplasmin oxidation system (Curzon & O'Reilly, 1960) and may perhaps be of some physiological significance.

SUMMARY

1. The oxidase activity of purified human caeruloplasmin was studied with *NN*-dimethyl-*p*-phenylenediamine as substrate by measuring the extinction of the red reaction product.

2. Anions caused inhibition in the order thiocyanate > fluoride > chloride > bromide > sulphate. The acetate buffer used also had some inhibitory effect.

3. Caeruloplasmin, which is partly inhibited by acetate or oxalate, is protected against inhibition by chloride.

4. Inhibition by chloride, and probably by acetate, is consistent with an anti-competitive mechanism.

5. The effects on activity of some chelating agents were studied. Ethylenediaminetetra-acetate (EDTA) inhibits non-competitively. The inhibition–log (EDTA concentration) curve, unlike the corresponding curves of oxalic acid and malonic acid, is sharply discontinuous.

6. Activity is increased by Fe^{2+} ions and to a lesser extent by Ni^{2+}, Co^{2+}, Zn^{2+} and Fe^{3+} ions at low concentrations. The same metals decrease activity at higher concentrations. Activity is also decreased by low concentrations of Hg^{2+}, Al^{3+} and In^{3+} ions.

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REFERENCES

Alberty, R. A. & Bock, R. M. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 895.

Broman, L. (1958). *Nature, Lond.*, **182**, 1655.

Burk, D., quoted by Ebersole, E. R., Guttentag, C. & Wilson, P. W. (1944). *Arch. Biochem.* **3**, 399.

Curzon, G. & O'Reilly, S. (1960). *Biochem. biophys. Res. Commun.* **2**, 284.

Curzon, G. & Vallet, L. (1960). *Biochem. J.* **74**, 279.

Dixon, M. & Webb, E. C. (1958). *Enzymes.* London: Longmans, Green and Co. Ltd.

Dodgson, K. S., Spencer, B. & Williams, K. (1955). *Biochem. J.* **61**, 374.

Dodgson, K. S., Spencer, B. & Williams, K. (1956). *Nature, Lond.*, **177**, 432.

Friedenwald, J. S. & Maengwyn-Davies, G. D. (1954). In *The Mechanism of Enzyme Action*, p. 163. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Holmberg, C. G. & Laurell, C. B. (1951). *Acta chem. scand.* **5**, 921.

Kistiakowsky, G. B. & Shaw, W. H. A. (1953). *J. Amer. chem. Soc.* **75**, 2751.

Mapson, L. W. (1941). *Biochem. J.* **35**, 1332.

Peacock, J. M. & James, J. C. (1951). *J. chem. Soc.* p. 2233.

Schales, O., Suthon, A. M., Roux, R. M., Lloyd, E. & Schales, S. S. (1948). *Arch. Biochem.* **19**, 119.

Scheinberg, I. H., Aisen, P. & Morell, A. G. (1959). *Vox. Sanguinis*, **4**, 69.

Scheinberg, I. H. & Morell, A. G. (1957). *J. clin. Invest.* **36**, 1193.

Sen, D. N., Mizushima, S., Curran, C. & Quagliano, J. V. (1955). *J. Amer. chem. Soc.* **77**, 211.

Vallee, B. L. (1955). *Advanc. Protein Chem.* **10**, 317.

Vogel, A. I. (1946). *A Textbook of Qualitative Chemical Analysis.* London: Longmans, Green and Co. Ltd.

Biochem. J. (1960) **77**, 74

1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and Rat Adipose Tissue

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The universal use of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT) and the knowledge that it readily accumulates within mammalian fat tissue clearly indicates that the health hazards of this insecticide cannot be ignored. Thus it has been demonstrated that DDT and its derivative, 1:1-di-(*p*-chlorophenyl)-2:2-dichloroethylene, can be found in the fat depots of humans who have had no occupational contact with the compounds (Hayes, Quinby, Walker, Elliott & Upholt, 1958). Further, they accumulate steadily during prolonged feeding experiments on man (Hayes, Durham & Cueto, 1956).

In recent years, experimental studies have shown that fat tissue, far from having a purely passive role of fat storage, is metabolically as active as most other tissues. In view of this active state it was of interest to examine the possibility that DDT *in situ* may affect the metabolism of fat tissue. In the present work, the behaviour of adipose tissue from rats which had been fed with DDT in their diet was examined. Other experiments are reported in which DDT was added to adipose tissue *in vitro*. The potential capacity of DDT and related compounds to influence cellular metabolism is shown by experiments in which these substances were added to rat-liver mitochondria.

EXPERIMENTAL

Male albino rats were used. Fat tissue was taken from the groin and epididymal regions and 'brown' fat was taken from the interscapular depot.

Composition of diet. Control diet was 41B powder (Bruce & Parkes, 1956) containing arachis oil added to a concentration of 1·3%.

DDT and Dieldrin diets were 41B powder containing 1·3% (v/w) of arachis oil in which had been dissolved DDT or Dieldrin to give final concentrations of 200 or 50 p.p.m. respectively.

Carbohydrate-rich diet was as described by Itzhaki & Wertheimer (1957), but with arachis oil as the fat component.

Special chemicals. 1:1:1-Trichloro-2:2-(*p*-chlorophenyl)-ethane, *pp'*-isomer, (DDT) was a gift of Geigy Co. Ltd.; for experiments with mitochondria this was recrystallized from 70% ethanol. 1:1-Di-(*p*-chlorophenyl)-2:2-dichloroethylene was prepared from DDT (Grumitt, Buck & Stearns, 1945). Di-(*p*-chlorophenyl) acetic acid was prepared from di-(*p*-chlorophenyl)dichloroethylene (Grumitt, Buck & Jenkins, 1945). 1:2:3:4:10:10-Hexachloro-6:7-epoxy-1:4:4a:5:6:7:8:8a-octahydro-1:4:5:8-dimethanonaphthalene (Dieldrin), a gift of Shell Petroleum Co. Ltd., London, was recrystallized from ethanol. 1:2:3:4:5:6-Hexachloro-*cyclo*hexane α-isomer, a gift of Imperial Chemical Industries Ltd., was also recrystallized from ethanol. Adrenaline acid tartrate, B.P., was obtained from Burroughs Wellcome and Co. Ltd., London, and insulin, 40 units/ml., from Allen and Hanbury Ltd., London. Bovine-plasma albumin, fraction V was supplied by The Armour Laboratories, Hampden Park, Eastbourne, Sussex.

Glycogen synthesis (Tuerkischer & Wertheimer, 1942.) One-month-old rats were fed for 3 months on a diet containing 200 p.p.m. of DDT. A second group of rats were fed on the control diet only. At the age of 4 months the rats were separated in individual cages and the quantity of food was restricted so that each rat received 6–7 g. of the appropriate diet daily. This weight of diet represented about 60% of the normal daily consumption. The rats had free access to water. At the end of 10 days the rats had lost approximately 20% of their previous weight. Two rats from each group were killed and samples of the groin-fat depots and of interscapular fat were taken for glycogen analysis (Somogyi, 1937). The rest of the rats were then given unrestricted access to 'carbohydrate-rich' diet until the end of the experiment. A number of rats (figures in parentheses, Fig. 1) from both the experimental and control groups were killed on each consecutive day of the refeeding period. Groin fat and interscapular fat were dissected from each animal and the glycogen content was determined. The results are expressed as g. of glucose/100 g. wet wt.

Glucose accumulation by epididymal fat. The rats were killed by a blow on the head and exsanguinated. The distal portion of each epididymal-fat pad was removed by a single cut, weighed on a torsion balance and dropped into a 10 ml. conical flask containing 1·6 ml. of Krebs–Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1959) containing 0·55M-glucose. To each flask was added 0·2 ml. of insulin or water. The final concentration of insulin was 2 units/ml. The flasks were sealed with Parafilm (A. G. Gallenkamp and Co. Ltd., London, E.C. 2) and then gassed for 10 min. with O_2+CO_2 (95:5) through syringe needles piercing the Parafilm. After gassing, the flasks were resealed with a second piece of Parafilm and shaken for 4 hr. at 37° in a Dubnoff metabolic shaker. Duplicate 0·25 ml. samples of medium were then taken for glucose

determination. The adipose tissue was removed from the medium, defatted and dried (Itzhaki & Wertheimer, 1957). Results are expressed as mg. of glucose taken up/10 mg. of dry defatted tissue/hr. In some experiments 20 mM-methylene blue replaced insulin.

Release of free fatty acids and the adrenaline effect. The initial stage of these experiments was as described in the preceding paragraph except for the composition of the incubation medium. Each 10 ml. conical flask contained 5 ml. of Krebs–Ringer phosphate solution (Umbreit *et al.* 1959) containing 1% of bovine albumin and adjusted to pH 7·4 (Gordon & Cherkes, 1958). Adrenaline (0·1 ml.) was added from a solution containing 50 μg./ml. After incubation at 37° for 4 hr. the adipose tissue was removed, defatted and dried as before. Samples of the medium were taken for analysis of free fatty acids. The results are expressed as μequiv. of fatty acid released/mg. of dry defatted tissue/hr.

Oxygen uptake. Oxygen uptake of fat tissue was measured in a conventional Warburg apparatus at 37° with air as the gas phase. Groin fat was removed as rapidly as possible after killing the animal and chilled on ice. Slices were cut on a Stadie–Riggs-type microtome, the lower part of which had been constructed of stainless steel. The microtome stood on a block of ice while the fat was sectioned. The fat slices were weighed on a torsion balance and placed in a Warburg flask containing 3·0 ml. of Krebs–Ringer phosphate solution, pH 7·4, with 0·01 M-glucose and 0·01 M-succinic acid; 0·15 ml. of 20% KOH was in the centre well. After an equilibration period of 10 min., O_2 consumption was measured at intervals for 3 hr. At the end of the experiment fat tissue was removed from the flasks and defatted and dried. Consumption of O_2 was expressed as μl. of O_2/mg. of dry defatted tissue/hr.

Each rat was decapitated and the brain quickly removed into ice-cold Krebs–Ringer phosphate solution. The cerebellum and hind brain were cut off, the hemispheres were divided and most of the white matter was removed from the lower surface. Two slices were cut with a Stadie–Riggs microtome, from each hemisphere, and placed directly into the main compartment of Warburg flasks. In each flask there was 3·0 ml. of Krebs–Ringer phosphate solution, pH 7·4, containing 0·01 M-glucose. Additions of DDT and of other compounds were made as 0·03 ml. of their solutions in dimethylformamide. Dimethylformamide (0·03 ml.) was added to the control tissues. The centre well contained 0·15 ml. of 20% KOH. The temperature was 37°. The flasks were gassed for 5 min. with O_2, equilibrated for 10 min. and manometric readings were taken for 60 min. The brain slices were then removed and dried on weighed watch glasses for 24 hr. at 105°. The results are expressed as μl. of O_2 uptake/mg. dry wt./hr.

Experiments with liver mitochondria. Liver mitochondria were prepared and experiments on oxidative phosphorylation, respiration in the presence of pyruvic acid and adenosine triphosphatase were performed as described by Aldridge (1958). Additions of DDT and other compounds were made as 0·03 ml. of their solutions in dimethylformamide and the same quantity of dimethylformamide was added to the control experiments.

Analytical methods. Glucose was determined by the method of Nelson (1944) as revised by Somogyi (1952). 1:1:1-Trichloro-2:2-di-(*p*-chlorophenyl)ethane was determined by the method of Schechter, Soloway, Hayes & Haller (1945) and unesterified fatty acids were determined by the method of Grossman, Palm, Becker & Moeller (1954).

RESULTS

The amount of DDT present in groin fat and interscapular fat was determined in three rats fed on a diet containing 200 p.p.m. of DDT for 3 months. The average concentrations of DDT in groin fat and in interscapular fat were 700 and 239 μg./g. respectively.

Wertheimer (1945) demonstrated that in rats which were semi-starved for several days and then given a carbohydrate-rich diet, there occurred a deposition of glycogen in the adipose tissue. This effect, although pronounced during the first 1 or 2 days of the refeeding régime, was transitory and was followed by fat deposition. As the change in fat and glycogen involved both synthetic and anabolic processes it might be sensitive to a toxic substance stored in the fat. This experiment was repeated on groups of DDT-fed and control rats. Comparison of the mean and standard error of the mean between DDT and normally fed rats on corresponding days of the refeeding period showed that DDT had no effect on the sequence or extent of the glycogen deposition (Fig. 1).

Most studies of the effect of DDT on oxidative metabolism have been performed on insect tissue and of the few concerned with mammalian tissue, in particular liver and brain slices, the results are variable and even contradictory (cf. Johnston, 1951). The effects *in vitro* on rat-brain-slice respiration of DDT, and of two other chlorinated insecticides, Dieldrin and 1:2:3:4:5:6-hexachloro*cyclo*hexane, are shown in Table 1. In the same table,

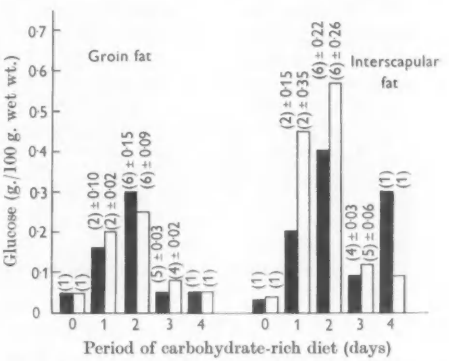

Fig. 1. Glycogen deposition in fat of rats fed on a carbohydrate-rich diet after a period of partial starvation. Glycogen is expressed as glucose. Figures in parentheses refer to number of rats and other figures are S.E.M. ■, Rats fed on 41 B diet containing 200 p.p.m. of DDT; □, rats fed on 41 B diet only.

the respiration of fat slices from DDT-fed rats is also recorded. There is no statistically significant difference between any of the experimental groups and the corresponding controls.

The metabolism of adipose tissue is influenced by insulin, both *in vivo* (Wertheimer, 1945) and *in vitro* (Itzhaki & Wertheimer, 1957; Winegrad & Renold, 1958). The last-named authors also describe experiments which suggest that, in adipose tissue, glucose may be metabolized by the phosphogluconate oxidative pathway. Brin & Yonomoto (1958), working with erythrocytes, demonstrated that the rate of glucose metabolism by this route could be stimulated by oxidation–reduction dyes such as methylene blue. In Table 2 are the results of experiments in which the glucose uptake of epididymal fat from DDT- and Dieldrin-fed rats was determined with and without the addition of insulin. The table also gives the result of adding methylene blue to the epididymal fat of DDT-fed rats. There was the expected increase of glucose uptake after the addition of insulin but the small increase after the addition of methylene blue was not significant. The presence of DDT or Dieldrin in the diet did not, under any condition, influence the uptake of glucose.

The factors influencing the mobilization of lipids from fat depots have been studied by Gordon (1957) and Dole (1956). The liberation of unesterified fatty acids from fat tissue *in vitro* was accomplished by Gordon & Cherkes (1958), who also demonstrated that the addition of adrenaline stimulated an increase of these acids. It was of interest to investigate the possibility that adipose tissue from DDT-fed rats might be different from normal fat in its response to adrenaline. Table 3 shows that the presence of DDT had no effect upon the release of fatty acids either in the presence or absence of adrenaline.

The preceding experiments indicate that metabolic activity of adipose tissue is insensitive to the presence of DDT. The ability of DDT and related substances to influence metabolic processes of cellular constituents is shown in the next experiment (Table 4). Also investigated were the main metabolites of DDT: 1:1-di-(*p*-chlorophenyl)-2:2-dichloroethylene and di-(*p*-chlorophenyl)acetic acid. All the compounds affect oxidative phosphoryl-

Table 1. *Effects of DDT, Dieldrin and hexachlorocyclohexane on respiration of tissue slices*

Q_{O_2} (μl. of O_2 uptake/mg. dry wt./hr.) is given with S.E.M. The last two columns give Student's t values and probabilities (P). Expt. 1, DDT fed to rats at 200 p.p.m. of diet for 3 months. Expt. 2, DDT and other insecticides added *in vitro*.

Compound	Tissue	Concentration	No. of rats	Q_{O_2} Control	Q_{O_2} Experimental	t	P
			Expt. 1				
DDT	Groin fat	200 p.p.m. in diet	5	9·2±2·6	6·2±2·6	1·1	> 0·3
			Expt. 2				
DDT	Brain	200 μM	9	9·8±0·5	10·2±0·3	0·7	> 0·5
Dieldrin	Brain	200 μM	4	7·9±2·3	11·4±4·2	0·7	> 0·5
Hexachloro-*cyclo*hexane	Brain	200 μM	2	9·2±1·4	10·8±1·1	0·9	> 0·5

Table 2. *Effect of insulin and methylene blue on glucose uptake of epididymal fat*

Results are given as mean values with S.E.M. Figures in parentheses are Student's t values and probabilities (P). Final concentrations were insulin, 2 units/ml., and methylene blue, 100 μM.

Expt.	No. of rats	No. of samples	Glucose uptake (mg. of glucose/10 mg. of dry defatted tissue/hr.) No insulin	Insulin
Control	3	11	1·42±0·36	2·94±0·40
DDT (200 p.p.m.)	3	12	1·15±0·13 (t 0·7, P > 0·4)	2·48±0·29 (t 0·9, P > 0·3)
Control	3	12	0·84±0·08	2·11±0·19
Dieldrin (50 p.p.m.)	3	12	1·07±0·11 (t 0·9, P > 0·3)	2·56±0·25 (t 0·8, P > 0·4)
			No methylene blue	Methylene blue
Control	3	12	0·63±0·18	1·03±0·17
DDT (200 p.p.m.)	3	12	0·79±0·07 (t 0·7, P > 0·4)	1·34±0·23 (t 1·1, P > 0·3)

Table 3. *Release of unesterified fatty acids from epididymal fat*

Epididymal fat (300–500 mg.) was incubated in 5 ml. of Krebs–Ringer phosphate (pH 7·4) containing 2% of bovine albumin. Adrenaline concn. was 1 μg./ml. of medium. Results are given as mean values with S.E.M. Figures in parentheses are Student's t values and corresponding probabilities (P) calculated by comparison of appropriate experiments.

Expt.	No. of rats	Adrenaline	Fatty acid released (μequiv./mg. of dry defatted tissue/hr.)
Normal diet	8	Absent	0·063 ± 0·0097
		Present	0·179 ± 0·027
DDT diet (200 p.p.m.)	6	Absent	0·081 ± 0·0097 (t 1·4, P 0·18)
		Present	0·203 ± 0·022 (t 0·7, P 0·5)

Table 4. *Concentrations of chlorinated insecticides and metabolites of DDT affecting liver mitochondria*

Oxidative phosphorylation: concentrations affecting inhibition of P uptake and O_2 uptake during oxidation of pyruvic acid. Oxidation: concentrations affecting oxidation of pyruvic acid in the absence of hexokinase and glucose. Adenosine triphosphatase (ATPase): concentrations producing maximal stimulation of adenosine triphosphatase in the absence of substrate. Figures in parentheses refer to μmoles of phosphate liberated/mg. of protein/hr. Control values were 1·03 ± 0·40 μmole/mg. of protein/hr. For details of reaction mixtures see Aldridge (1958).

		Oxidative phosphorylation			
Compound	Concn. (μM)	Inhibition of phosphate uptake (%)	Inhibition of oxygen uptake (%)	Oxidation (μM)	ATPase (μM)
DDT	35	62	64	40 (67% stimulation)	200 (7·3)
Dieldrin	35	62	49	—	—
Hexachloro*cyclo*hexane	90	50	37	— (Inhibition)	—
Di-(*p*-chlorophenyl) acetic acid	80	55	37	40 (77% stimulation)	40 (5·2)
Di-(*p*-chlorophenyl)-dichloroethylene	40	31	24	40 (50% stimulation)	200 (7·3)

ation but DDT differs from the others in that O_2 uptake is inhibited to the same extent as P uptake, so that the oxidative phosphorylation quotient (P/O) remains unaltered. The other compounds inhibit P uptake more than O_2 uptake and thus to some extent lower the P/O ratio. DDT and its metabolites stimulate respiration of mitochondria with pyruvate as substrate in the absence of hexokinase and glucose. Dieldrin had no effect and hexachloro*cyclo*hexane inhibited respiration. DDT and di-(*p*-chlorophenyl)dichloroethylene stimulated adenosine triphosphatase activity at concentrations more than 10 times as high as the concentrations affecting oxidative phosphorylation.

DISCUSSION

The accumulation of DDT in fat tissue has been shown in animals (Laug, Nelson, Fitzhugh & Kunze, 1950) and in humans (Hayes *et al.* 1958). The greatest amount of DDT found in the fat of humans who were not deliberately exposed to DDT was 50 p.p.m. (Pearce, Mattson & Hayes, 1952). By including DDT in the diet of volunteers for 1 year, Hayes *et al.* (1956) were able to increase this figure to an average of 340 p.p.m. without ill effect. This level can be compared with that of 700 p.p.m., the concentration in fat of male rats fed with DDT at 200 p.p.m. During the experimental period these rats showed no symptoms of DDT poisoning. Evidence that the DDT is stored in a pharmacologically active form is derived from an earlier experiment in female rats. After a period of DDT feeding followed by a few days of semi-starvation these rats exhibited symptoms of DDT poisoning and most of them died. It is known that female rats store nearly twice as much DDT as male rats (Ortega, Hayes, Durham & Mattson, 1956).

The experiments described in this paper do not reveal any deletorious effects of DDT on the metabolism of adipose tissue. The concentration of DDT (700 p.p.m.) in groin fat of rats fed with DDT for

3 months is equivalent to a concentration of approximately 2 mM. The experiments of Johnston (1951) and those reported here on liver mitochondria show that this concentration is much higher than that necessary to affect intracellular enzymic processes. The insensitivity of the enzymes of fat cells may be due to the protective effect of the fat within the cells. Johnston (1951) has demonstrated that the effect of DDT on the succinoxidase system is greatly reduced if the DDT is added in an oil emulsion instead of in an ethanol solution. The lack of influence of DDT on a variety of enzymes studied by Judah (1949) can also be attributed to the use of an oil emulsion as the vehicle by which DDT was added to the enzyme systems. This objection was met in the present experiments by using dimethylformamide, a solvent which is miscible with water. The protection *in vivo* afforded by fat in relationship to drug action has been emphasized by Brodie & Hogben (1957). These authors describe experiments in which a short-acting barbiturate, *N*-methylthiopentone, which has a high distribution coefficient in favour of lipid, was given in repeated doses to dogs. After each recovery from anaesthesia the dose could be repeated a number of times without affecting the depth or duration of anaesthesia. This the authors attribute to fat acting as a seemingly bottomless reservoir for the drug.

It is concluded that fat not only protects the whole animal from possible effects of DDT but that it also protects fat cells themselves even when the metabolism of these cells is increased. There is no evidence that the amounts of DDT likely to accumulate in human fat present any toxicological hazard.

SUMMARY

1. The deposition of glycogen in rat adipose tissue, which occurs after refeeding partially starved rats, was not affected by previously feeding the rats on 1:1:1-trichloro-2:2-di(*p*-chlorophenyl)-ethane (DDT) at 200 p.p.m.

2. Previous feeding of DDT did not affect the respiration *in vitro* of groin-fat slices or the glucose uptake and unesterified fatty acid release by epididymal fat. The glucose uptake of epididymal fat was unaffected by the presence of 1:2:3:4:10:10-hexachloro-6:7-epoxy-1:4:4 a:5:6:7:8:8 a-octahydro-1:4:5:8-dimethanonaphthalene (Dieldrin) in the diet.

3. Oxidation of glucose by brain slices occurred at normal rates in the presence of DDT, Dieldrin or 1:2:3:4:5:6-hexachloro*cyclo*hexane. In contrast, enzymic activities of rat-liver mitochondria were modified by the addition *in vitro* of DDT, Dieldrin, hexachloro*cyclo*hexane or two metabolites of DDT.

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REFERENCES

Aldridge, W. N. (1958). *Biochem. J.* **69**, 367.
Brin, M. & Yonomoto, R. H. (1958). *J. biol. Chem.* **230**, 307.
Brodie, B. B. & Hogben, C. A. M. (1957). *J. Pharm., Lond.*, **11**, 345.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
Dole, V. P. (1956). *J. clin. Invest.* **35**, 150.
Gordon, R. S. (1957). *J. clin. Invest.* **36**, 810.
Gordon, R. S. & Cherkes, A. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 150.
Grossman, M. I., Palm, L., Becker, G. H. & Moeller, H. C. (1954). *Proc. Soc. exp. Biol., N.Y.*, **87**, 312.
Grumitt, O., Buck, A. & Jenkins, A. (1945). *J. Amer. chem. Soc.* **67**, 155.
Grumitt, O., Buck, A. & Stearns, J. (1945). *J. Amer. chem. Soc.* **67**, 156.
Hayes, W. J., jun., Durham, W. F. & Cueto, C., jun. (1956). *J. Amer. med. Ass.* **162**, 890.
Hayes, W. J., jun., Quinby, G. E., Walker, K. C., Elliott, J. W. & Upholt, W. M. (1958). *A.M.A. Arch. industr. Hlth*, **18**, 398.
Itzhaki, S. & Wertheimer, E. (1957). *Endocrinology*, **61**, 72.
Johnston, C. D. (1951). *Arch. Biochem. Biophys.* **31**, 375.
Judah, J. D. (1949). *Brit. J. Pharmacol.* **4**, 120.
Laug, E. P., Nelson, A. A., Fitzhugh, O. G. & Kunze, F. (1950). *J. Pharmacol.* **98**, 268.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Ortega, P., Hayes, W. J., jun., Durham, W. F. & Mattson, A. M. (1956). *Publ. Hlth Monogr.*: *Publ. Hlth Serv. Hlth, Educ. & Welf. Dep., U.S.A.*, **43**, 1.
Pearce, G. W., Mattson, A. M. & Hayes, W. J., jun. (1952). *Science*, **116**, 254.
Schechter, M. S., Soloway, S. B., Hayes, R. A. & Haller, H. L. (1945). *Industr. Engng Chem.* (*Anal.*), **17**, 704.
Somogyi, M. (1937). *J. biol. Chem.* **117**, 711.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Tuerkischer, E. & Wertheimer, E. (1942). *J. Physiol.* **100**, 385.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1959). *Manometric Techniques*, 3rd ed. p. 149. Minneapolis: Burgess Publishing Co.
Wertheimer, E. (1945). *J. Physiol.* **103**, 359.
Winegrad, A. I. & Renold, A. E. (1958). *J. biol. Chem.* **233**, 267, 273.

The Determination of Tungsten in Biological Material by Activation Analysis

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Tungsten exists at such low concentrations in normal biological material that it has been detected only in tissues from animals previously fed with unusually large quantities of tungsten (Kinard & Van de Erve, 1941), or plants growing in tungsten-rich soils (J. P. R. Polkinhorne, personal communication). In view of the discovery that the related element molybdenum is essential for plants and animals and is a constituent of at least two enzyme systems, it would seem that further measurements of tungsten might yield equally interesting results. The present note describes a technique capable of detecting as little as 1 μmg. of tungsten.

The method used is that of activation analysis, which is now established as an extremely sensitive technique for determining trace elements in tissue (Loveridge & Smales, 1957; Bowen & Cawse, 1959). A number of workers have studied the distribution of tungsten in samples of geochemical and metallurgical interest by this technique (e.g. Atkins & Smales, 1959; Leliart, Hoste & Eeckhaut, 1959), and a recent paper describes the determination of the element in various marine organisms (Fukai & Meinke, 1959). Tungsten has a relatively high cross-section for thermal neutrons (much higher than that of molybdenum) and, among the radioactive isotopes produced, 24-hr. ^{187}W is much the most abundant. In this work chemical methods were used to isolate the ^{187}W activity from the other radioelements occurring in the activated material. Contamination before activation was minimized as described in the next section, and subsequent contamination would not affect the analysis.

EXPERIMENTAL

Tomato plants were grown in sand culture and seeds collected as described by Bowen & Cawse (1959). Flax plants were grown in soil in a glasshouse. The top parts of these plants were air-dried and ground in a specially made Perspex rotary mill. This mill consisted of a Perspex propeller rotating at 3000 rev./min. in a hollow cylindrical chamber 12·5 cm. in diameter. Nuts were commercial samples of unknown history. About 1 g. of dry tissue, which was handled with plastic apparatus only, was used in each analysis. Blood samples were collected from healthy donors at a collection centre; 1–2 ml. was allowed to run into weighed polythene ampoules, which had been cleaned with aqua regia. This blood had been in contact with a stainless-steel hypodermic needle and a short length of rubber tubing, so cannot be considered completely free from contamination. The blood samples were weighed, dried under cover at 80° and sealed in their containers. Tissue samples were sealed in small polythene bags and activated together with a standard consisting of 100 μg. of tungsten absorbed on a single dry tomato seed ('ashless' filter paper was later found to be equally suitable as an absorbent). The standard was delivered from a micrometer syringe attached to a polythene pipette. Standard solutions were made by careful dilution of a stock solution of spectroscopically pure tungsten trioxide in distilled ammonia. Activation was carried out in the Harwell reactor BEPO in a flux of about 10^{12} thermal neutrons/cm.^{2}/sec. for a 24 hr. period.

Chemical separation of tungsten. The separation scheme evolved is original but owes much to the work of Prestwood (1958). Two of the major activities generated in the tissues, ^{24}Na and ^{42}K, were easily removed by simple chemical processes, but a third, ^{32}P, generally co-precipitated with tungsten and proved much more difficult to eliminate. The largest activities generated in trace elements were calculated to be 12 hr. ^{64}Cu and 2·6 hr. ^{56}Mn (Bowen, 1956).

The activated sample or standard was first partially wet-ashed by boiling with 5–10 ml. of 16 N-nitric acid containing 20 mg. of tungsten as sodium tungstate in a 50 ml. beaker. No hold-back carriers were employed. After wet-ashing for a few minutes, the contents of the beaker were washed into a 50 ml. centrifuge tube and yellow tungsten trioxide was spun down. The supernatant liquid was poured off, the tungsten trioxide was dissolved in 2 ml. of 15 N-ammonia and reprecipitated by boiling with 10 ml. of 16 N-nitric acid. This cycle was repeated and scavenging precipitations of ferric hydroxide and magnesium ammonium phosphate were carried out at pH 8 while the tungsten was in solution. Sufficient 50% ammonium acetate was added to buffer the solution, and 1 drop of 10% ferric nitrate, 2 drops of N-ammonium dihydrogen phosphate and 4 drops of N-magnesium chloride were added successively before centrifuging. The supernatant liquid was poured into a 50 ml. separating funnel containing 10 ml. of chloroform, 4 ml. of 5% cupferron and 1 drop of 10% (w/v) copper sulphate pentahydrate. After acidifying with 50% tartaric acid, the funnel was shaken and the chloroform layer was run off. A second shaking with fresh chloroform was used to remove elements such as copper, which form cupferrates soluble in this solvent. The remaining aqueous layer was poured into a fresh 50 ml. centrifuge tube and about 10 ml. of 24 N-nitric acid added carefully till the tartaric acid was oxidized

and the tungsten precipitated as the trioxide. This precipitate was spun down, dissolved in 2 ml. of 15 N-ammonia and a further scavenging precipitation made with magnesium ammonium phosphate in the presence of ammonium acetate. The clear supernatant from this step was treated with 2 ml. of 2% α-benzoin-oxime and made 6 N with respect to nitric acid. It was necessary to carry out this cycle of scavenging and α-benzoin-oxime precipitation two or three times to achieve satisfactory decontamination from ^{32}P. The final precipitate was washed three times with water, slurried on to an aluminium counting tray with acetone, dried and heated in a muffle furnace for 30 min. at 540° to convert the oxime into tungsten trioxide, which was weighed to determine the chemical yield. The average chemical yield was about 50%: the count relative to the standard gave the amount of tungsten in the sample. ^{187}W emits a β ray of 1·31 Mev energy, and was counted with an end-window Geiger counter surrounded by an anticoincidence shield to reduce the background to 1·5 counts/min. No self-absorption correction was found to be necessary. The whole chemical procedure took about 3 hr. for eight samples.

Counting was carried out at 24 hr. intervals to establish the radiochemical purity of the separated ^{187}W. In all cases where residual activity was found after 10 days it was shown to be caused by 14-day ^{32}P.

Losses in the chemical steps were measured radiochemically for four replicates, in order to improve the efficiency of separation and the overall chemical yield. In addition to the steps described above, the following were tested: (*a*) scavenging ammonium tungstate at pH 8 by precipitating copper sulphide, (*b*) extracting tungsten α-benzoinoxime into chloroform and (*c*) re-extracting the latter complex into N-sodium hydroxide; these were not used in the actual analysis, but are included in Table 1 for comparison. The extraction of tungsten α-benzoinoxime into chloroform would have been a useful decontamination step, had it not been so inefficient with the quantities of tungsten carrier used. The sulphide-precipitation step might be necessary for samples containing large amounts of copper.

RESULTS

The recovery of tungsten added to seeds or filter paper in amounts less than 1 μg. was reasonably good. In Fig. 1 the weights of tungsten taken are plotted against the counts/min. obtained in the final precipitate, corrected for chemical yield, and it can be seen that the curve is linear. Since 0·1 μg. of tungsten yielded about 3000 counts/min. and the background of the counter was 1·5 counts/min.,

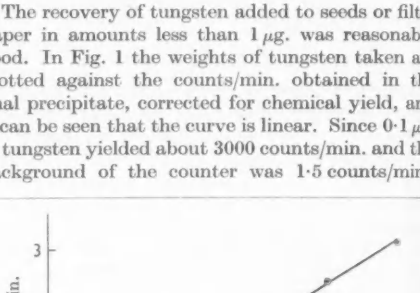

Fig. 1. Recovery of added tungsten from tomato seeds.

Table 1. *Losses of tungsten during chemical separation after activation*

The steps are described in detail in the text.

Chemical step	Loss (%)	No. of times used per analysis
WO_3 ppt. from 12 N-HNO_3	1·5	4
Tungsten α-benzoinoxime ppt. from 6 N-HNO_3	5·6	2
$CHCl_3$ extraction of tungsten cupferrate	3·0	1
$CHCl_3$ extraction of tungsten α-benzoinoxime	16·4	0
Re-extraction of tungsten α-benzoinoxime into N-NaOH	3·4	0
$Fe(OH)_3$ scavenging ppt. at pH 8	3·5	1
$MgNH_4PO_4$ scavenging ppt. at pH 8	3·4	3
CuS scavenging ppt. at pH 8	3·3	0

Table 2. *Tungsten in plant tissues*

Species	Tissue	Origin	Tungsten found (μmg./g. dry wt.)
Arachis hypogaea (pea nut)	Seed	Commercial	2·6, 9·0
Bertholletia excelsa (Brazil nut)	Seed	Commercial	7·3, 16·7
Castanea sativa (chestnut)	Seed	Commercial	1·6, 3·1
Corylus avellana (hazel nut)	Seed	Commercial	0·45, 0·46
Juglans regia (walnut)	Seed	Commercial	7·3, 11·0
Lycopersicon esculentum (tomato)	Seed	Commercial*	150
L. esculentum (tomato)	Seed	Sand culture + Mo	140
L. esculentum (tomato)	Seed	Sand culture – Mo	23
L. esculentum (tomato)	Top parts	Sand culture	52, 58
Linum usitatissimum (flax)	Top parts	Greenhouse culture	71, 84, 92

* Suttons, Reading.

the amount of tungsten needed to double the background was 50 $\mu\mu$g. This is not the ultimate limit of detection, since neutron fluxes much larger than those used in this work are now available, which would extend the sensitivity still further.

The amounts of tungsten found in the plant tissues studied are shown in Table 2.

Nuts were chosen for analysis because of their relative freedom from surface contamination. The variation found in the commercial samples is not surprising. Only the last two entries show the reproducibility of the method, as here samples were taken from homogeneous powdered tissue: the errors to be expected are of the order of $\pm 10\%$. The molybdenum contents of the tomato seeds grown in sand culture with and without added molybdenum were known to be 1900 and 210 μmg./g. respectively (Bowen, 1959).

Tungsten was also determined in human blood samples from two adult males and seven females: the results were: males, 0·75–1·20 μmg. of tungsten/g.; females, 0·46–2·34 μmg. of tungsten/g. The mean value of all the samples was $1{\cdot}09 \pm 0{\cdot}51$ μmg. of tungsten/g., or 0·103 μg. of tungsten/100 ml. Previous work indicates that the molybdenum content of mammalian blood is about 16 μmg./g. (Bowen, 1959).

DISCUSSION

Atkins & Smales (1959) have discussed the errors involved in activation analysis for tungsten and have concluded that spurious results might be obtained if large amounts of rhenium or osmium were present relative to tungsten. Unfortunately the content of these elements in plant tissue and blood has never been measured, but it is unlikely to have affected the present results. There are no tungsten isotopes among the fission products, so fission of any uranium in the samples during activation would not interfere.

There seems little doubt that the method described here is very much more sensitive and more selective than other techniques which have been used to determine tungsten in biological material. Colorimetric techniques (Aull & Kinard, 1940; Ward, 1951; Allen & Hamilton, 1952; Jeffery, 1953) cannot be used to detect less than 1 μg. of tungsten, and are more or less subject to interference by molybdenum. The first restriction means that the techniques cannot be used to determine the amounts of tungsten occurring in normal biological tissue, and the second is equally serious since it seems likely that molybdenum is 10–100 times more abundant than tungsten in both biological and geological material (see below). Interference from molybdenum has not been troublesome in this type of analysis, as it has a much lower cross-section for thermal neutrons than tungsten, and it is also effectively removed by the chemical procedure.

There are virtually no figures in the literature with which to compare the present analyses. Recently, Fukai & Meinke (1959) have found 0·13–0·18 μg./g. of ash in a marine alga by activation, which corresponds to roughly 15 μmg./g. dry wt. Our results are of the same order of magnitude. The figures for blood, though small, vary only by a factor of 5. This constancy does not necessarily mean that tungsten is an essential element, since the results may simply reflect the solubility of stainless-steel hypodermic needles in blood, or indicate the tolerable level of a trace contaminant which the kidneys are not able to remove. Normal steels contain about 0·2% of tungsten (Atkins & Smales, 1959), and the solubility of the element in blood is not known. Hypodermic needles are slightly soluble in water, and it was shown by weighing on a microbalance that a needle lost 174 μg. after 12 l. of water had passed through it. This would correspond to a solubility of 0·03 μmg. of tungsten/ml. The limited data available support the hypothesis that the tungsten/molybdenum ratio in biological material may be largely determined by its environment: in the earth and in terrestrial organisms the ratio is approximately 1:10, whereas in the sea and in marine organisms it is nearer 1:100 (Kuroda & Sandell, 1954; Ishibashi, Shigematsu & Nakagawa, 1954; Atkins & Smales, 1959; Bowen, 1959; Fukai & Meinke, 1959). Molybdenum is known to be an essential element for living creatures, and many studies have shown it to be accumulated by them. The present results indicate that tungsten is accumulated to about the same extent, showing that it, too, may be essential. There are already a number of reports in the literature on the stimulating effects of tungsten on plant growth (Scharrer & Schropp, 1934; Young, 1935; Krisciunas, 1936; Arnon, 1938; Hoagland, 1941; Davies & Stockdill, 1956). Further studies of this problem, with controlled ratios of molybdenum to tungsten in the culture medium, would appear to be warranted. Tungsten does not become toxic until it equals or exceeds molybdenum in abundance (Higgins, Richert & Westerfield, 1956). The method of activation analysis has sufficient sensitivity to measure the element at concentrations well below the toxic level.

SUMMARY

1. A method is described for determining tungsten in biological material by neutron-activation analysis. The theoretical sensitivity is 50 $\mu\mu$g. of tungsten, and in practice 1 μmg. can be determined with an accuracy of $\pm 10\%$.

2. Tungsten was determined in various plant

tissues and found to be present in amounts from 0·5 to 150 μmg./g. dry wt.

3. The metal was also determined in nine samples of human blood and found to be present in amounts from 0·5 to 2·3 μmg./g. of whole blood, with a mean of 1·1 μmg./g. (0·10 μg./100 ml.).

4. The limited data on tungsten/molybdenum ratios indicate that tungsten, though much less common than molybenum, is accumulated to an approximately equal extent from the environment by living organisms.

REFERENCES

Allen, S. H. & Hamilton, M. B. (1952). *Analyt. chim. acta*, **7**, 483.

Arnon, D. I. (1938). *Amer. J. Bot.* **25**, 322.

Atkins, D. H. F. & Smales, A. A. (1959). *Atomic Energy Research Establishment, Report* no. R. 3099. London: H.M.S.O.

Aull, J. C. & Kinard, F. W. (1940). *J. biol. Chem.* **135**, 119.

Bowen, H. J. M. (1956). *J. Nuclear Energy*, **3**, 18.

Bowen, H. J. M. (1959). *Int. J. appl. Radiat. Isotopes*, **5**, 227.

Bowen, H. J. M. & Cawse, P. A. (1959). *Atomic Energy Research Establishment, Report* no. R. 2925. London: H.M.S.O.

Davies, E. B. & Stockdill, S. M. J. (1956). *Nature, Lond.*, **178**, 866.

Fukai, R. & Meinke, W. W. (1959). *Nature, Lond.*, **184**, 815.

Higgins, E. S., Richert, D. A. & Westerfield, W. W. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 509.

Hoagland, D. R. (1941). *Proc. Amer. Soc. hort. Sci.* **38**, 8.

Ishibashi, M., Shigematsu, T. & Nakagawa, Y. (1954). *Bull. Inst. phys. chem. Res., Japan*, **32**, 199.

Jeffery, P. G. (1953). *Rep. Geol. Dep. Uganda*, **75**, 1.

Kinard, F. W. & Van de Erve, J. (1941). *J. Pharmacol.* **72**, 196.

Krisciunas, J. (1936). *Zemes, Ukis.* no. 19–20.

Kuroda, P. K. & Sandell, E. B. (1954). *Geochim. et cosmoch. Acta*, **6**, 35.

Leliart, G., Hoste, J. & Eeckhaut, J. (1959). *Talanta*, **2**, 115.

Loveridge, B. A. & Smales, A. A. (1957). *Meth. biochem. Anal.* **5**, 225.

Prestwood, R. J. (1958). *U.S. Atomic Energy Commission, Los Alamos*, Rep. LA-1721 (2nd Rev.). Ed. by J. Kleinberg *et al.* Washington D.C.: Office of Technical Services.

Scharrer, K. & Schropp, W. (1934). *Z. PflErnähr. Dung.* **34**A, 312.

Ward, F. N. (1951). *Circ. U.S. geol. Surv.* 119.

Young, R. S. (1935). *Rep. N.Y. agric. Exp. Sta.* **174**, 2.

Biochem. J. (1960) **77**, 82

The Phospholipids of Ox Spleen with Special Reference to the Fatty Acid and Fatty Aldehyde Compositions of the Lecithin and Kephalin Fractions

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The fatty acid compositions of lecithin and kephalin fractions from different animal tissues have not yet been studied in a systematic manner, though several excellent papers have been published by Klenk and his co-workers (Klenk & Bohm, 1951; Klenk, Debuch & Daun, 1953; Klenk & Krickau, 1957) on the whole glycerophosphatide fraction and some purified phospholipids from a number of animal tissues. From their results it is apparent that different tissues contain lecithin and kephalin fractions which differ widely in their fatty acid composition.

The plasmalogens differ from the classical ester phosphatides in having only one fatty acid esterified with the glycerol moiety instead of two. Instead of the other fatty acid there is a carbon chain of similar length attached to the glycerol by an $\alpha\beta$-unsaturated ether linkage. This linkage is easily ruptured by weak acid to give a long-chain fatty aldehyde and a lysophosphatide. It has been shown that in ox brain (Debuch, 1958) and ox heart (Debuch, 1958; Gray, 1958) the fatty acids of the choline and ethanolamine plasmalogens are mostly unsaturated. Is this a characteristic difference between the plasmalogens and ester phosphatides which is common to all tissues?

Much less is known of the fatty aldehyde composition of plasmalogens from different tissues. Stearaldehyde and palmitaldehyde are the main components of aldehyde mixtures isolated from ox-heart muscle (Klenk, Stoffel & Eggers, 1952; Gray, 1958) and 50% of the aldehydes isolated from

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ox-brain tissue have been identified as unsaturated compounds, mostly with one double bond (Debuch, 1958). Aldehydes from the plasmalogens shown to be present in many other tissues have not been identified. A systematic study of the distribution patterns of fatty acids and fatty aldehydes of the lecithin and kephalin fractions separated quantitatively from selected tissues has been started. In this paper those isolated from ox spleen are compared with those of ox heart, ox liver, pig heart and ram semen.

The term kephalin is used for mixtures of phosphatidylethanolamine, phosphatidylserine and the corresponding plasmalogens (referred to separately as kephalin plasmalogen).

EXPERIMENTAL AND RESULTS

Analytical methods

The estimations of P, total N, amino N, ethanolamine and serine, choline, aldehyde, inositol, fatty acid ester groups and iodine value were carried out as described by Gray & Macfarlane (1958).

Extraction and fractionation of the phospholipids from ox spleen

The extraction was based on that of Folch, Ascoli, Lees, Meath & LeBaron (1951). Ox spleen (365 g. wet wt.), obtained 2–3 hr. after death, was cut up and blended for 30 sec. in an MSE blender at room temperature with 5 vol. of chloroform–methanol (1:1, v/v) and kept for 15–20 min. in a large jar with occasional stirring. The extract was filtered on a Büchner funnel and the residue re-extracted twice with 1 vol. of chloroform–methanol (2:1, v/v). The extracts were combined and poured into a 5 l. jar (solvent volume approx. 2·7 l.). The solution was washed by stirring with 1 vol. of tap water; after 1–2 hr. the water was siphoned off and kept. A red–brown interfacial solid was removed from the surface of the chloroform and two more washings were carried out with 0·5 vol. (approx. 1 l.) of water; these washings were combined with the first one. The chloroform solution was kept at −10° overnight to freeze out the remaining water, filtered at 2° and the solution dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* at 35° and the lipid dissolved in light petroleum (b.p. 40–60°). Non-P lipid (e.g. neutral fat, cholesterol, sterol esters) was removed from the phospholipids by the dialysis method of van Beers, de Iongh & Boldingh (1958). This method is most useful for removing non-P lipid with the advantage of quantitatively retaining all the phospholipids. After dialysis the light-petroleum solution of ox-spleen phospholipids was evaporated to dryness at 35° *in vacuo* and redissolved in chloroform. The analysis of this solution is given in Table 1. No inositide was found in the chloroform extract. The aqueous washings, saved from the extraction procedure, were evaporated to dryness at 35° *in vacuo* and the residue was extracted with ether and chloroform–methanol (2:1, v/v). The solvents were evaporated off and the residue was dissolved in a known volume of chloroform. Analysis gave total P, 1·2 mg.; inositol was not detected. The presence of small amounts of phosphatidylinositol cannot be excluded as it is possible that the substance was not extracted by organic solvents from the large amount of inorganic salts, nucleotides, amino acids etc., present in the residue owing to occlusion or the formation of a complex.

Table 1. *Analysis of total phospholipid extract of ox spleen*

Values given are those of 365 g. (wet wt.) of ox spleen. The percentage of P (on dry-weight basis) was 3·98.

	Amount present (mg.)	Molar ratio (P = 1)
Total P	228·0	1
Total N	124·0	1·19
Amino N*	46·5	0·45
Serine N†	21·6	0·21
Ethanolamine N†	27·5	0·27
Choline N	51·2	0·50
Aldehyde	406·0	0·23
Ester P‡	200·6	0·88
Fatty acid ester groups	—	1·73

* Estimated by the ninhydrin method of Lea & Rhodes (1954).

† Estimated by the method of Axelrod, Reichenthal & Brodie (1953).

‡ Estimated by the method of Schmidt, Benotti, Hershman & Thannhauser (1946).

Fractionation of phospholipids on silicic acid. The phospholipids as a 10% solution in chloroform–methanol (49:1, v/v) were chromatographed on a column (diameter, 31 mm.) containing 160 g. of silicic acid in chloroform–methanol (49:1, v/v). Elution was started with the same solvent. A fast-running yellow-coloured fraction containing no P was quickly eluted from the column and found to be mainly free aldehydes, which indicated the probable breakdown of some plasmalogen. This fraction was closely followed by another small P-free fraction which was probably a trace of neutral fat not removed during the previous dialysis procedure. The concentration of methanol in chloroform was gradually increased as the different phospholipid fractions were eluted from the column. Fig. 1 illustrates the separation curve obtained based on analysis of the fractions for P, amino N and fatty acid ester groups. Approximately 90% (193 mg.) of the total P loaded on to the column was recovered. An attempt to recover the remainder by increasing the methanol concentration in chloroform to 90% was unsuccessful so the solvent was changed to chloroform–methanol–water (1:4:1, by vol.) and finally to chloroform–methanol–aq. 2N-NH_3 soln. (1:4:1, by vol.). The last solvent eluted a further fraction (H) containing 8 mg. of P. Table 2 shows the analysis of the bulked fractions A–H.

Nature of the phospholipids in the various fractions

Acidic phospholipids. A small fast-moving band (fraction A), low in amino N and very unsaturated, was eluted with chloroform–methanol (19:1, v/v). It was presumably substantially cardiolipin (Gray & Macfarlane, 1958).

Diacyl phosphatides and plasmalogens. Fractions B, C and D were mixtures of phosphatidylethanolamine, ethanolamine plasmalogen, phosphatidylserine and serine plasmalogen. Fraction B was eluted as a very sharp band with chloroform–methanol (9:1, v/v) and contained 88% of serine phosphatides and only 4% of ethanolamine phosphatides. The plasmalogens accounted for 7% of the total P in the fraction. Fraction B also contained approx. 10% of non-amino-N-containing material, which was most probably more cardiolipin trailing from fraction A. The separation of a fast-running serine fraction is unusual and may have been due to a relatively high acidity, either of the original phospholipid solution or of the silicic acid column, and the presence of some phosphatidylserine in the free acid form. Fraction C was a small band containing a mixture of diacyl phosphatides, plasmalogens and probably the remainder of the cardiolipin. Fraction D contained approx. 80% of ethanolamine phosphatides and 20% of serine phosphatides. Plasmalogens account

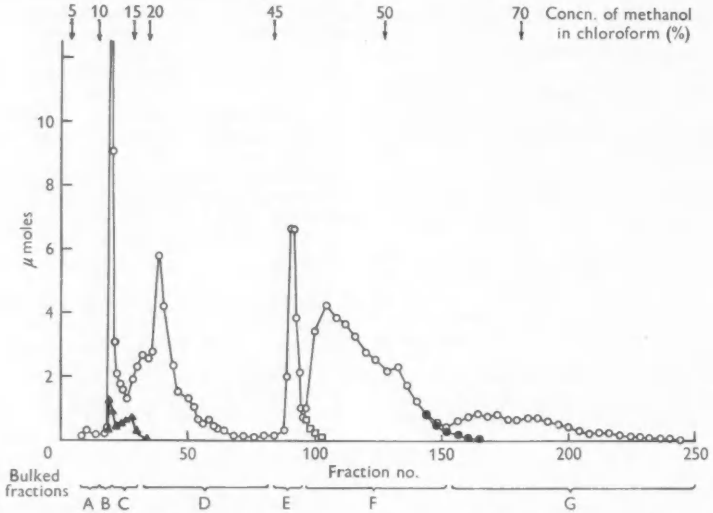
5 10 15 20 45 50 70 Concn. of methanol in chloroform (%)
μ moles
0 2 4 6 8 10
0 50 100 150 200 250
Fraction no.
Bulked fractions
A B C D E F G

Fig. 1. Fractionation of the phospholipid extract from ox spleen on silicic acid. ○, Total P; ▲, P present in N-free phospholipid, calculated from P and amino N values; □, amino N; ●, ester P, calculated from fatty acid ester group:P ratio.

Table 2. *Distribution and analysis of phospholipids separated by silicic acid chromatography*

The total P on the column was 215 mg. and the total P recovered was 201 mg.

			Molar ratio (P = 100)					
Fraction (see Fig. 1)	Tubes	P content (% of total lipid P)	Amino N*	Ethanolamine N†	Serine N†	Aldehyde	Fatty acid ester group	Double bonds
A	8–15	0·6	6	—	—	Nil	210	320
B	16–20	7·1	89	4	88	7	206	99
C	21–30	5·8	64	—	—	21	190	163
D	31–83	17·2	107	80	23	32	179	166
E	84–95	11·2	85	63	16	9	100	102
F	97–151	36·8	2	Nil	Nil	11	190	214
G	152–252	11·0	Nil	Nil	Nil	4	20	95
H	253–	3·7	98	38	21	Nil	145	296

* By the ninhydrin method of Lea & Rhodes (1954). † By the method of Axelrod *et al.* (1953).

for 32% of the total P. Fraction F was a mixture of phosphatidylcholine (89%) and choline plasmalogen (11%).

Lysophosphatides. Fraction E was mainly lysophosphatidyl-ethanolamine and -serine from the breakdown of the ethanolamine and serine plasmalogens. It was slightly contaminated with lecithin from fraction F.

Sphingomyelin. Fraction G was substantially sphingomyelin. The molar ratio P:N of 1:1·91 (theory 1:2) and the presence of 10% of ester P, estimated by the method of Schmidt, Benotti, Hershman & Thannhauser (1946), showed that it was contaminated with lecithin from the previous fraction F (Fig. 1).

Other components. Fraction H, eluted with some difficulty from the column, was not examined in detail. It had a P:N ratio of 1:1·7; P, 2·6% on a dry wt. basis. Apparently some ethanolamine and serine (Table 2) were present, but since the total amino N was much higher than the sum of these two compounds it is possible that the method used (Axelrod, Reichenthal & Brodie, 1953) was not estimating either, but some other amino N compound. Contamination of the material with ammonia from the eluting solvent was ruled out because the fraction had been evaporated to dryness at 35° *in vacuo*, dissolved in ether and extracted with 2N-HCl before analysis. The presence of unsaturated fatty acids was indicated by the high ester value and iodine value (double bonds, Table 2). It is not known whether the fraction was homogeneous or a mixture of fragments which had been very strongly adsorbed on the silicic acid.

Composition of ox-spleen phospholipid. From the analyses in Table 2 and the results in Table 3 the distribution of phospholipids was computed (as percentage of the total lipid P) as: cardiolipin (and similar compounds), 3·0; phosphatidylethanolamine, 9; phosphatidylserine, 14; ethanolamine plasmalogen, 10; serine plasmalogen, 5; phosphatidylcholine, 35; choline plasmalogen, 4; sphingomyelin, 10; unidentified, 4; not recovered, 6%.

Fatty acids and fatty aldehydes in the kephalin and lecithin fractions

The fatty acids and aldehydes were isolated from the kephalin, kephalin plasmalogen, lecithin and choline plasmalogen of ox spleen and from the following phospholipid fractions of other tissues: the kephalin and lecithin fractions of ox heart, pig heart (Gray & Macfarlane, 1958) and ox liver (prepared by Dr M. G. Macfarlane) and the lecithin fraction of ram semen (Gray, 1960*a*). The procedures used for the ox-spleen fractions are applicable to the kephalin and lecithin fractions of any tissue.

The isolation of the fatty acids from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine and the fatty acids and aldehydes from the ethanolamine, serine and choline plasmalogens is carried out by first treating the mixed kephalin and mixed lecithin fractions with 90% acetic acid at 38° for 18 hr. (Gray, 1958). Under these conditions the aldehydes split off from the

Table 3. *Separation of the products of 90%-acetic acid hydrolysis of the kephalin fraction on silicic acid*

Fraction	Eluting solvent $CHCl_3$–methanol (ratio, v/v)	Tubes	Total P (mg.)	Molar ratio: P:fatty acid ester groups	Molar ratio: Ethanolamine:serine	Comment
(*a*)	49:1	1–10	—	—	—	Free aldehydes
(*b*)	19:1	11–20	4·8	1:2	—	Contained only 18% of amino N
(*c*)	9:1	21–30	19·7	1:2	1:3	Diacyl phosphatide
(*d*)	9:1	31–40	1·96	1:1·2	—	Lysophosphatidic acid
(*e*)	4:1	41–65	12·6	1:2	1:0·69	Diacyl phosphatide
(*f*)	4:1	66–80	2·4	1:1·9		
(*g*)	11:9	81–100	19·5	1:1	1:0·51	Lysophosphatide
(*h*)	11:9	101–140	3·3	1:1		

Table 4. *Separation of the products of 90%-acetic acid hydrolysis of the lecithin fraction on silicic acid*

Fraction	Eluting solvent $CHCl_3$–methanol (ratio, v/v)	Tubes	Total P (mg.)	Molar ratio (P:fatty acid ester groups)	Comment
(i)	4:1	1–7	—	—	Free aldehydes
(ii)	1:1	8–50	65·0	1:1·98	Phosphatidylcholine
(iii)	1:1	51–73	1·17	1:1·63	
(iv)	1:1	74–91	0·45	1:1·64	
(v)	3:7	92–140	5·55	1:1·07	Lysophosphatidylcholine

Table 5. *Fatty acid composition of different phospholipids from various tissues*

The shortened designation (column 2) was evolved by Ahrens *et al.* (1959); e.g. br 16:1 represents a branched mono-unsaturated C_{16} compound; hbr, probably highly branched; un, unknown; tr, trace.

		Ox spleen		Ox liver		Ox heart		Pig heart	
Familiar name of acid	Shortened designation	Lecithin	Choline plasmalogen	Lecithin	Choline plasmalogen	Lecithin	Choline plasmalogen	Lecithin	Choline plasmalogen
Capric	10:0	—	—	—	—	—	—	—	—
Undecanoic	11:0	tr	—	—	tr	—	—	—	—
	br 12:0	—	—	tr	0·1	—	—	—	—
Lauric	12:0	tr	0·3	tr	0·2	—	—	—	—
Tridecanoic	13:0	—	—	—	—	—	—	—	—
Myristic	14:0	0·6	0·3	tr	0·2	0·4	tr	0·1	tr
Myristoleic	14:1	—	—	—	—	—	—	—	—
	hbr 15:0	0·3	—	—	—	—	—	—	—
	br 15:0	tr	—	0·2	tr	—	—	—	—
Pentadecanoic	15:0	1·2	0·9	0·2	tr	0·5	tr	0·1	—
	br 16:0	1·0	0·3	—	—	0·4	tr	0·2	0·7
Palmitic	16:0	45·0	37·8	12·7	14·0	36·7	6·9	26·4	8·3
Palmitoleic	16:1	1·6	1·7	0·6	0·9	4·2	2·3	1·5	0·6
	un	—	—	—	—	—	—	—	—
	hbr 17:0	0·9	1·1	—	—	—	—	—	—
	br 17:0	2·4	2·3	0·6	0·3	1·4	—	—	—
Heptadecanoic	17:0	1·4	1·5	0·7	0·8	1·0	—	0·4	—
	17:1	—	—	—	0·2	1·5	1·0	—	—
	17:2	—	—	—	—	—	—	—	—
	br 18:0	—	—	—	—	tr	2·0	—	—
Stearic	18:0	16·3	14·0	35·0	21·9	8·7	2·5	9·3	1·7
Oleic	18:1	19·1	17·3	14·3	12·1	30·0	36·4	25·8	26·6
*iso*Oleic	*iso* 18:1	—	tr	1·6	3·0	—	—	—	—
Linoleic	18:2	6·1	7·1	7·7	4·7	14·5	42·4	25·1	53·6
Linolenic	18:3	—	tr	tr	0·7	1·3	2·5	tr	0·9
	un	—	—	—	—	—	—	—	—
	un	—	—	—	tr	—	—	—	—
	20:3	0·9	0·6	3·5	3·0	—	1·8	—	—
Arachidonic	20:4	3·5	13·6	7·8	8·5	—	2·4	3·0	7·9
	20:5	—	—	2·6	3·9	—	tr	—	tr
	un	—	—	0·6	1·2	—	—	—	—
	un	—	—	1·2	—	—	—	—	—
	22:5	—	—	9·6	18·3	—	—	—	—
	22:6	—	—	1·5	5·9	—	—	—	—

	22:6	—	—	1·5	5·9	—	—	—	—	

Table 5 (*cont.*)

		Ram semen		Ox spleen		Ox liver		Ox heart		
Familiar name of acid	Shortened designation	Lecithin	Choline plasmalogen	Kephalin	Kephalin plasmalogen	Kephalin	Kephalin plasmalogen	Kephalin	Kephalin plasmalogen	Pig-heart kephalin
Capric	10:0	—	—	—	tr	—	—	tr	0·5	—
Undecanoic	11:0	0·3	0·6	—	—	—	—	tr	0·2	—
	br 12:0	—	—	—	—	—	—	tr	0·7	—
Lauric	12:0	0·2	0·8	0·2	tr	0·1	0·2	tr	0·9	tr
Tridecanoic	13:0	—	—	—	tr	—	—	—	—	—
Myristic	14:0	5·0	5·8	0·2	0·2	tr	0·2	0·2	0·3	0·2
Myristoleic	14:1	0·4	1·0	—	—	—	—	—	—	—
	hbr 15:0	—	—	—	—	0·1	—	—	—	—
	br 15:0	0·8	1·2	—	—	0·1	tr	tr	tr	—
Pentadecanoic	15:0	1·9	2·3	—	0·2	0·1	0·1	tr	tr	0·2
	br 16:0	0·5	2·2	—	—	0·1	tr	—	2·1	1·4
Palmitic	16:0	37·9	32·0	5·8	6·0	12·3	15·2	3·5	7·8	11·1
Palmitoleic	16:1	3·1	5·4	0·4	0·4	0·3	0·5	1·2	0·8	0·6
	un	2·8	tr	—	—	—	—	—	—	—
	hbr 17:0	—	—	—	—	—	—	—	—	—
	br 17:0	—	—	—	—	1·0	0·7	0·8	2·1	—
Heptadecanoic	17:0	0·9	1·6	0·6	1·3	1·2	1·2	1·0	—	0·8
	17:1	16·2	10·1	0·6	1·3	—	—	—	—	—
	17:2	4·3	2·2	—	—	—	—	—	—	—
	br 18:0	—	—	—	—	—	—	—	—	0·5
Stearic	18:0	8·2	11·7	63·0	43·0	39·4	39·5	58·0	12·1	29·0
Oleic	18:1	6·5	9·5	21·4	12·7	13·2	13·9	6·5	15·8	11·9
*iso*Oleic	*iso* 18:1	tr	tr	—	—	6·5	8·5	2·9	tr	—
Linoleic	18:2	0·9	tr	4·3	2·8	} 6·8	} 4·35	13·6	40·7	25·2
Linolenic	18:3	—	—	—	—			0·9	3·3	tr
	un	4·2	2·9	—	—	tr	0·4	—	—	—
	un	4·2	—	—	—	—	—	—	—	—
	20:3	tr	—	tr	1·9	1·3	1·5	tr	4·9	tr
Arachidonic	20:4	1·9	11·5	6·9	29·2	} 11·1	} 9·4	11·6	7·1	18·6
	20:5	—	—	tr	1·5			tr	0·8	—
	un	—	—	—	—	—	—	—	—	—
	un	—	—	—	—	—	—	—	—	—
	22:5	—	—	—	—	5·1	3·8	—	—	—
	22:6	—	—	—	—	1·1	0·6	—	—	—

plasmalogen with the formation of the lysophosphatide, but the ester phosphatides are not attacked. The free aldehydes, the diacyl phosphatide and the lysophosphatide are easily separated by chromatography on silicic acid and the fatty acids are obtained from the phosphatide fractions by mild alkaline hydrolysis.

Kephalin fraction. Fractions B, C, D and E (Table 2) were bulked and a volume containing 70 mg. of P was evaporated to dryness. The residue was dissolved in 7 ml. of 90% (v/v) acetic acid and incubated at 38° for 18 hr. The solution was cooled, neutralized with NaOH and then shaken with an equal volume of chloroform–methanol (2:1, v/v). The phases were separated by centrifuging and the upper aqueous methanol layer was removed. The chloroform layer was washed three times with 0·2 vol. of 0·5M-NaCl. The presence of the NaCl was necessary to keep emulsion formation to a minimum. The chloroform solution was dried over anhydrous Na_2SO_4, filtered and evaporated at 35° *in vacuo*; the residue was redissolved in a known volume of chloroform–methanol (49:1, v/v). The total P in the chloroform solution was 66·5 mg. and the water-soluble P (in aqueous methanol extract) was 3·2 mg.

The phospholipid extract was chromatographed on 65 g. of silicic acid in chloroform–methanol (49·1, v/v) and the results are shown in Table 3. The aldehydes in fraction (*a*) were added to those collected from the original separation after breakdown of some of the kephalin plasmalogens (as described previously), and the combined aldehydes were converted into their dimethylacetal derivatives with the usual precautions (Gray, 1960*b*). Fraction (*b*) was probably a mixture of cardiolipin [present in fractions B and C (Table 2)] and some phosphatidic acid, formed from the ester phosphatide by a slight loss of base during the 90%-acetic acid hydrolysis. Fractions (*c*), (*e*) and (*f*) contained the diacyl phosphatides, (*c*) being rich in phosphatidylserine (cf. fraction C, Table 2). Fractions (*g*) and (*h*) contained the lysophosphatidylethanolamine and lysophosphatidylserine.

Fractions (*c*), (*e*) and (*f*) were combined and the solvent was removed by evaporation at 35° *in vacuo*. The residue (34 mg. of P) was dispersed in 50 ml. of aqueous N-NaOH and saponified in a stoppered flask under N_2 at room temperature overnight. Fractions (*g*) and (*h*) were combined (22·5 mg. of P) and saponified in 30 ml. of aqueous N-NaOH overnight. The mixtures were acidified with 5N-HCl and the fatty acids were extracted with ether. The ethereal solutions were washed with water and dried over anhydrous Na_2SO_4. The ether was evaporated off and the mixtures of acids were refluxed for 3 hr. with 5–10 vol. of anhydrous methanolic 0·8N-HCl and quantitatively converted into their methyl esters for analysis by gas–liquid chromatography.

Lecithin fraction. A major portion (74 mg. of P) of fraction F, the lecithin fraction containing 11% of choline plasmalogen, was treated with 7 ml. of 90% acetic acid as described previously. Water-soluble P, found after hydrolysis, was 1·23 mg. and chloroform-soluble P was 72·2 mg. The chloroform solution of phospholipids and free aldehydes was chromatographed on 60 g. of silicic acid in chloroform–methanol (4:1, v/v) and the results are shown in Table 4.

Fraction (i) containing the aldehydes was dried and the aldehydes were converted into the dimethylacetals. Portions of the phosphatidylcholine [30 mg. from fraction (ii)] and of the lysophosphatidylcholine [fraction (v), P, 5·5 mg.] were saponified and the acids were isolated and converted into their methyl esters.

Gas–liquid chromatography. The chromatographic apparatus was a laboratory-constructed model, with the argon β-ray-ionization detector designed by Lovelock, James & Piper (1959). All columns were of glass, 4 ft. long and with internal diameter of 4 mm. The stationary phases used were Apiezone L grease (1·8 g./7 g. of Celite) and Reoplex 400 (2·1 g./7 g. of Celite). The Celite (100–120 mesh) was prepared by the method of James & Martin (1952) and for use with Apiezon L grease was pretreated with ethanolic alkali (James, Martin & Smith, 1952).

Major acids were identified by comparison with retention volumes of pure standards. The presence of unsaturated components was confirmed by bromination and their degree of unsaturation assessed by the method of James (1959). The separation and identification of the fatty aldehydes has been described in detail elsewhere (Gray, 1960*b*). The fatty acid composition of the ester phosphatides and the fatty acid and aldehyde composition of the plasmalogens of ox spleen are shown in Tables 5 and 6 and are compared with similar components from ox heart, ox liver, pig heart and ram semen.

DISCUSSION

The results obtained from previous investigations (Debuch, 1958; Gray, 1958) suggested that most of the fatty acids in plasmalogens would be unsaturated. The results obtained from the study of five different tissues (Tables 5 and 7) do not, however, support this idea. The acids from the plasmalogens from ox and pig hearts are mostly unsaturated but the plasmalogens of ox spleen, ox liver and ram semen have widely varying proportions of saturated and unsaturated acids. The major unsaturated acids in all the phosphatide fractions with the exception of those from ox liver are oleic

Table 6. *Aldehyde composition of plasmalogens from different tissues*

For abbreviations see Table 5.

Familiar name of aldehyde	Shortened designation	Ox spleen		Ox heart		Ox liver		Pig heart		Ram semen
		Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen	Kephalin plasmalogen	Choline plasmalogen
Capryl	10:0	—	0·3	—	—	—	—	—	0·8	—
	11:0	—	—	—	—	tr	tr	—	0·8	0·5
Lauryl	12:0	0·1	0·4	0·2	—	tr	tr	0·1	—	—
	hbr 13:0	0·2	—	—	—	—	—	—	—	—
	br 13:0	0·4	—	—	—	0·1	—	—	—	—
Tridecanal	13:0	0·5	—	—	—	0·6	—	—	—	—
	un	—	—	—	—	0·5	—	—	—	—
	br 14:0	7·3	1·1	1·4	0·6	7·4	5·1	—	0·4	0·9
Myristyl	14:0	4·8	0·9	0·7	1·0	3·4	4·2	—	0·3	1·0
Myristoleyl	14:1	—	—	0·2	—	—	—	—	—	—
	hbr 15:0	3·0	1·1	3·9	1·1	8·3	6·0	0·60	1·8	10·5
	br 15:0	20·9	1·5	2·9	2·3	27·3	23·6	—	tr	2·3
Pentadecanal	15:0	6·7	2·1	3·4	3·2	4·6	6·7	0·7	1·9	8·2
	un	—	—	—	—	—	—	—	—	2·1
	un	—	—	—	—	—	—	—	—	1·3
	br 16:0	7·2	1·3	1·8	—	9·5	5·3	1·2	—	—
Palmityl	16:0	29·7	31·6	67·5	33·3	21·6	20·0	82·8	44·0	61·3
Palmitoleyl	16:1	1·2	—	1·7	—	—	5·0	—	—	1·1
	hbr 17:0	6·0	1·7	2·0	—	1·4	6·1	—	1·3	tr
	br 17:0	5·4	2·4	3·9	3·9	2·5	6·3	1·1	0·9	4·0
Heptadecanal	17:0	1·1	4·4	1·5	3·9	1·5	1·9	2·6	2·8	0·7
	un	—	—	—	—	3·2	7·4	—	—	—
	un	—	—	—	—	—	—	—	—	3·2
	br 18:0	0·9	—	—	—	—	—	—	—	—
Stearyl	18:0	4·7	42·8	9·0	42·6	4·5	11·1	6·3	34·5	2·0
Oleyl	18:1	—	4·0	tr	6·9	0·7	—	4·8	10·5	1·3
	iso 18:1	—	2·40	—	—	—	—	—	tr	—
	un	—	—	—	—	1·8	3·9	—	—	—
	un	—	—	—	—	—	1·4	—	—	—
Linoleyl	18:2	—	2·3	tr	1·3	—	—	tr	—	—
Linolenyl	18:3	—	—	—	—	—	—	—	—	—

acid, linoleic acid and arachidonic acid, higher proportions of the last being found in the kephalin and kephalin plasmalogen fractions. Linolenic acid occurs only in very small amounts. The ox-liver phosphatides also contain substantial amounts of eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid (cf. Klenk & Montag, 1957; Klenk & Tomuschat, 1957). Fatty acids with odd-numbered carbon chains and branched-chain acids only occur in small or trace amounts in all fractions except those from ram semen, which apparently contain substantial amounts of heptadecanoic acids with one and two double bonds. It is necessary to examine ram-semen phosphatides from a number of different sources before accepting these acids as normal components. With the exception of ox liver the major saturated acid in the lecithins and in the choline plasmalogens is palmitic acid (C_{16}) whereas the major saturated acid in the kephalins and in the kephalin plasmalogens is stearic acid (C_{18}). This distribution of saturated C_{16} and C_{18} acids may be a characteristic pattern in phosphatides from the majority of tissues.

The distribution patterns of the aldehydes (Table 6) are very complicated. Debuch (1958) found that 50% of the total aldehydes from the ethanolamine plasmalogen of ox brain were unsaturated, but the aldehydes from the plasmalogens studied in this work contain only small proportions of mono-unsaturated components. Surprising features are the large proportions of branched-chain aldehydes in ox spleen and ox liver. The most common aldehyde of this type appears to be a branched-chain C_{15} compound; it is a major component of the aldehydes from ox-spleen choline plasmalogen and the major component of the aldehydes from ox-liver plasmalogens. The chemical structures of these compounds are not yet known, but a structural similarity to the branched-chain acids (Table 5) is to be expected.

The distribution of C_{16} and C_{18} fatty acids, in different phosphatide fractions, is paralleled by the distribution of C_{16} and C_{18} aldehydes. In all cases (ox liver excepted) the proportion of palmitaldehyde (C_{16}) in choline plasmalogen is much greater than that in kephalin plasmalogen from the same tissue. In the same way the proportion of stearaldehyde (C_{18}) in the kephalin plasmalogen is much greater than that in the choline plasmalogen. If the patterns in these few tissues are representative of most tissues they suggest that in the biosynthesis of lecithin/choline plasmalogen and kephalin/kephalin plasmalogen there is an enzymic preference for saturated fatty acids and aldehydes of 16 and 18 carbon atoms respectively. Whether or not this is true might be verified by using the enzyme systems described by Kennedy (1957) for the biosynthesis of lecithin and kephalin starting with model diglycerides containing the appropriate fatty acids.

Table 7. *Approximate amounts of unsaturated fatty acids present in different phospholipid fractions*

	Fraction as percentage of total phospholipid in tissue	Unsaturated acids in fraction (%)
1. Lecithin		
Ox spleen	35	31
Ox heart	18	51
Ox liver	58	50
Pig heart	27	55
Ram semen	36	33
2. Choline plasmalogen		
Ox spleen	4	40
Ox heart	24	90
Ox liver	6	72
Pig heart	23	89
Ram semen	36	43
3. Kephalin		
Ox spleen	23	34
Ox heart	25	38
Ox liver	26	45
Pig heart	27	56
4. Kephalin plasmalogen		
Ox spleen	15	50
Ox heart	18	73
Ox liver	6	43

SUMMARY

1. Phospholipids equivalent to about 600 mg. of phosphorus/kg. of fresh tissue were extracted from ox spleen with chloroform–methanol mixtures and were fractionated on silicic acid columns.

2. The percentage composition of the phospholipid was: cardiolipin, 3; phosphatidylethanolamine, 9; phosphatidylserine, 14; ethanolamine plasmalogen, 10; serine plasmalogen, 5; phosphatidylcholine, 35; choline plasmalogen, 4; sphingomyelin, 10; unidentified, 4; not recovered, 6.

3. The fatty acids of the ester phosphatides and the fatty acids and aldehydes of the corresponding plasmalogens were isolated after treatment of the mixed phosphatide fractions with 90% acetic acid at 38° and separation of the products on silicic acid.

4. The composition of the various fatty acid and fatty aldehyde mixtures from the phosphatide fractions of ox spleen were determined by gas–liquid chromatography and the distribution patterns were compared with those of similar fractions from ox heart, ox liver, pig heart and ram semen.

5. Large amounts (approx. 50%) of branched-chain aldehydes are present in ox-spleen choline plasmalogen and ox-liver plasmalogens. A branched-chain C_{15} compound is a major component of the aldehydes from both these tissues.

6. The results suggest a selective incorporation of saturated fatty acids and aldehydes of 16 carbon atoms into lecithin and choline plasmalogen and of 18 carbon atoms into kephalin and kephalin plasmalogen.

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REFERENCES

Ahrens, E. H., Insull, W., Hirsch, J., Stoffel, W., Peterson, M. L., Farquhar, J. W., Miller, T. & Thomasson, H. J. (1959). *Lancet*, i, 115.

Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.

Debuch, H. (1958). In *Essential Fatty Acids*, p. 48. Ed. by Sinclair, H. M. London: Butterworths Scientific Publications.

Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951). *J. biol. Chem.* **191**, 833.

Gray, G. M. (1958). *Biochem. J.* **70**, 425.

Gray, G. M. (1960*a*). *Biochem. J.* **74**, 1P.

Gray, G. M. (1960*b*). *J. Chromat.* **4**, 52.

Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.

James, A. T. (1959). *J. Chromat.* **2**, 552.

James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

James, A. T., Martin, A. J. P. & Smith, G. Howard (1952). *Biochem. J.* **52**, 238.

Kennedy, E. P. (1957). *Annu. Rev. Biochem.* **26**, 119.

Klenk, E. & Bohm, P. (1951). *Hoppe-Seyl. Z.* **288**, 98.

Klenk, E., Debuch, H. & Daun, H. (1953). *Hoppe-Seyl. Z.* **292**, 241.

Klenk, E. & Krickau, G. (1957). *Hoppe-Seyl. Z.* **308**, 98.

Klenk, E. & Montag, W. (1957). *Liebigs Ann.* **604**, 4.

Klenk, E., Stoffel, W. & Eggers, J. J. (1952). *Hoppe-Seyl. Z.* **290**, 246.

Klenk, E. & Tomuschat, H. J. (1957). *Hoppe-Seyl. Z.* **308**, 165.

Lea, C. H. & Rhodes, D. N. (1954). *Biochem. J.* **56**, 613.

Lovelock, J. E., James, A. T. & Piper, E. A. (1959). *Ann. N.Y. Acad. Sci.* **72**, 720.

Schmidt, G., Benotti, J., Hershman, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.

van Beers, G. J., de Iongh, H. & Boldingh, G. (1958). In *Essential Fatty Acids*, p. 43. Ed. by Sinclair, H. M. London: Butterworths Scientific Publications.

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Studies in the Biosynthesis of Fungal Metabolites

THE BIOSYNTHESIS OF PALITANTIN

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Palitantin, $C_{14}H_{22}O_4$, a metabolite of *Penicillium palitans* Westling and other species, was first isolated and characterized by Birkinshaw & Raistrick (1936). It was further investigated by Birkinshaw (1952) and by Bowden, Lythgoe & Marsden (1959), who proposed the structure (I) (Fig. 1).

The biosynthesis of this C_{14}-alicylic compound is of interest in its relation to auroglaucin (II) and flavoglaucin, phenolic products of *Aspergillus glaucus* (Gould & Raistrick, 1934; Quilico, Cardani & Lucattelli, 1953), in that they each contain a C_1 and a linear C_7 side chain attached to adjacent carbon atoms of a C_6 ring. Also auroglaucin has been shown by Birch, Schofield & Smith (1958) to be derived by the condensation of acetate units. Since the formation of aromatic rings by this mechanism may well involve alicyclic intermediates the possible relevance of palitantin biosynthesis is readily apparent.

Although acetate coupling is the pathway most readily visualized (Bowden *et al.* 1959), the presence of three vicinal oxygen substituents is reminiscent of 5-dehydroshikimic acid (III), which is a known precursor of the aromatic amino acids and consequently an alternative source of benzenoid rings. Although the necessary additional modifications render this latter mechanism less probable, the possible operation of such a pathway added interest to the problem of palitantin biosynthesis.

The approach used in this investigation was based on the incorporation of labelled precursors into palitantin and subsequent determinations of the resulting distribution of radioactive carbon atoms. Thus a palitantin-yielding strain of *Penicillium cyclopium* Westling was grown in surface culture on Czapek-Dox medium and its rate of production determined (Fig. 2). The peak yield occurred at about 16 days and an attempt was

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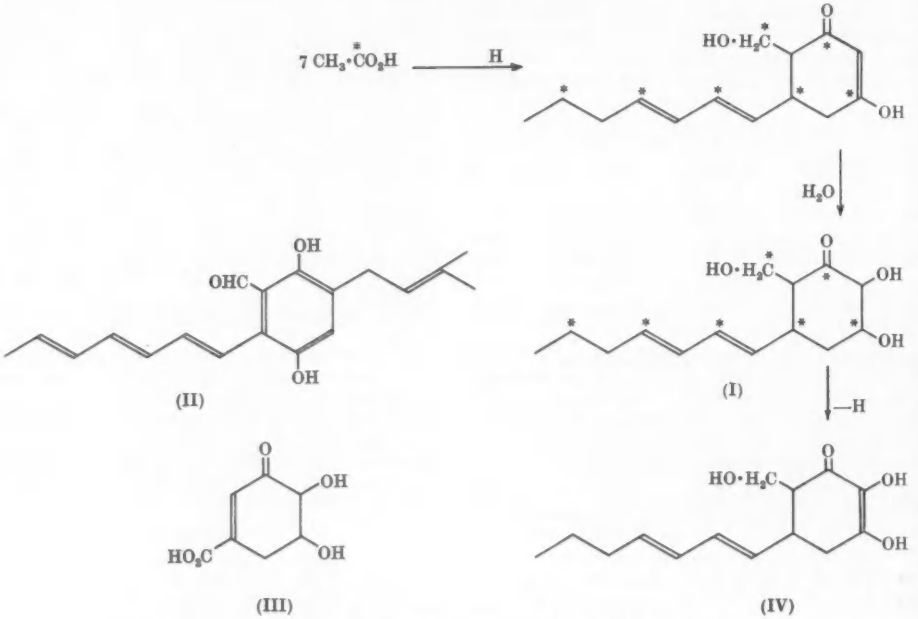

Fig. 1. Suggested pathway of palitantin biosynthesis. Asterisks indicate carbon atoms derived from acetate carboxyl groups.

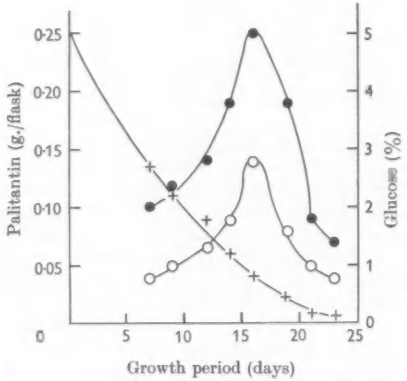

Fig. 2. Palitantin production. Each flask contains 350 ml. of culture medium. +, Glucose; ●, crude palitantin; ○, crude frequentin.

made to prepare labelled palitantin by adding sodium [1-^{14}C]acetate to the medium after 8 days' growth and subsequent ether extraction of the metabolism solutions of the individual flasks after 11, 13 and 15 days' growth.

The resulting extract was fractionated with sodium carbonate solution into crude palitantin and frequentin, $C_{14}H_{20}O_4$, a related product of possible structure (IV) (Bowden *et al.* 1959). Both fractions were highly labelled, as shown in Table 1.

As only single flasks were tested it is not possible to make any significant observations about the influence of incubation time on the incorporation of acetate. However, it is apparent in each case that the activity of the palitantin fraction appreciably exceeded that of the frequentin fraction. This result suggests that palitantin may be a precursor of frequentin, which in terms of structures (I) and (IV) would involve a single dehydrogenation step. However, the lower activity of the crude frequentin fraction could also result from the presence of unlabelled impurities.

The crude palitantin fractions were combined, purified and subsequently diluted with inactive material in order to produce sufficient product for the degradations outlined in Fig. 3.

The results of these degradations are given in Table 2 and compared with the theoretical values based on the incorporation of seven acetate units into palitantin according to the scheme shown in Fig. 1.

Table 1. *Incorporation of labelled acetate and mevalonic acid lactone when added to single flasks containing 8-day cultures of* Penicillium cyclopium

Each flask contained 350 ml. of culture fluid (for details see text).

Labelled precursor	Sodium acetate 40 μc of [1-^{14}C]-labelled			Mevalonic acid lactone 10 μc of [2-^{14}C]-labelled	Mevalonic acid lactone 25 μc of [1-^{14}C]-labelled
Growth (days)	11	13	15	11	11
Glucose (%)	1·32	1·00	0·95	1·51	1·44
Mycelial weight (g.)	2·11	2·10	2·02	2·24	2·40
Activity of mycelium powder (counts/min.)					
Before extraction	764	693	957	22	4
After extraction	419	485	482	—	—
Light petroleum extract					
Weight (g.)	0·137	0·054	0·081	0·108	0·112
Activity (counts/min.)	5991	3563	8863	147	26
Ether extract					
Weight (g.)	0·098	0·062	0·064	0·076	0·078
Activity (counts/min.)	1255	1384	1932	140	29
Crude palitantin					
Weight (g.)	0·207	0·094	0·151	0·142	0·149
Activity (counts/min.)	7722	4986	5227	0	0
Incorporation of added acetate into palitantin (%)	15	4·4	5·7	—	—
Crude frequentin					
Weight (g.)	0·081	0·071	0·102	—	—
Activity (counts/min.)	5539	3735	3640	—	—

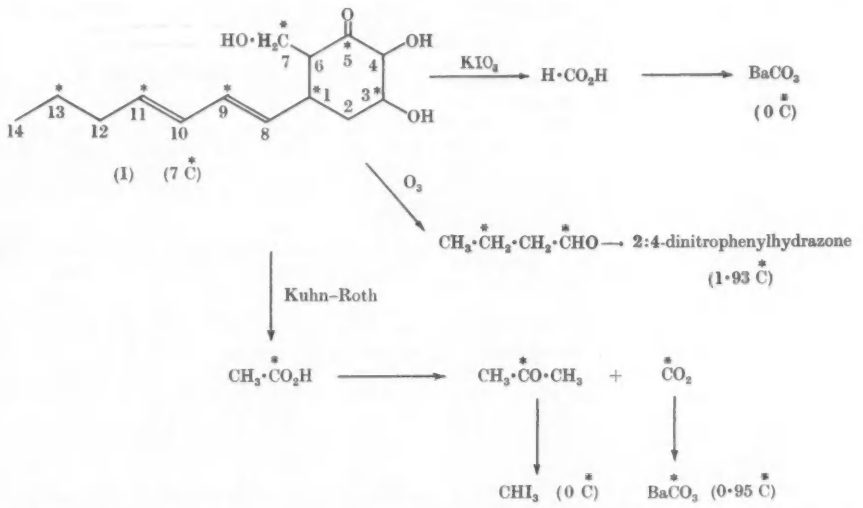

Fig. 3. Palitantin degradation scheme. The activities of the degradation products (shown in parentheses) are calculated on the basis of a theoretical incorporation of seven acetate units into palitantin.

Table 2. *Distribution of labelling in palitantin derived from* [1-^{14}C]*acetate*

Carbon atom in palitantin	Radioactivity (counts/min.)	Radioactivity (μc/mole)	Active carbon equivalent Found	Active carbon equivalent Required
1–14 as palitantin	641	611	—	7*
14 (as CHI_3)	0	0	0	0
13 (as $BaCO_3$)	112	83	0·95	1
11, 12, 13, 14 (as *n*-butyraldehyde 2:4-dinitrophenylhydrazone)	178	168	1·93	2
11, 12 (by difference)	—	—	0·98	1
4 (as $BaCO_3$)	0	0	0	0

* Based on theoretical incorporation of seven acetate units.

Thus a Kuhn–Roth oxidation isolated C-13 and C-14 as acetic acid. This was converted by pyrolysis of its lithium salt into acetone and subsequently iodoform, which corresponded to C-14 of palitantin and was inactive. The residual lithium carbonate (from C-13) contained an activity of 0·95 unit when assayed as barium carbonate.

A periodate oxidation isolated C-4 as formic acid, which on oxidation to carbon dioxide and assay as barium carbonate or on direct assay as lead formate was inactive.

Ozonolysis of palitantin yielded *n*-butyraldehyde, which was assayed as its 2:4-dinitrophenylhydrazone. The activity (1·93 units) corresponded to the aggregate of C-11, C-12, C-13 and C-14 from which it followed, by difference, that C-11 and C-12 shared an activity of 0·98 unit.

These results are in good agreement with the values required by the acetate head-to-tail condensation mechanism. Further experiments to confirm the presence of alternate labelling throughout the molecule were discontinued at this stage, when it was learnt from Professor A. J. Birch, F.R.S., that an investigation of the biosynthesis of palitantin had recently been completed in his Department at the University of Manchester. He kindly informed us of his findings, which agreed with those described above and consequently confirmed the validity of the postulated pathway from acetate.

The hydroxymethyl side chain is apparently derived from a reduced acetate carboxyl and this also occurs in the biosynthesis of citromycetin (Birch, Fitton *et al.* 1958). In auroglaucin, a parallel reduction of the acetate carboxyl-derived C_1 side chain also takes place but proceeds only as far as the aldehyde. No evidence is available to indicate the stage at which this reduction takes place but one possibility involves the acetate-derived, isoprenoid precursor, mevalonic acid (V). In this case the incorporation would not involve the usual decarboxylation stage (Fig. 4).

Accordingly, [2-^{14}C]mevalonic acid lactone (10 μc) and also the [1-^{14}C]-labelled lactone (25 μc) were fed in separate experiments to *P. cyclopium* under conditions shown to be conducive to acetate uptake. However, in neither case was the resulting palitantin significantly labelled. The utilization by the fungus of the added mevalonic acid was tested by determining the activity of the crude lipoid fraction obtained from extraction of the mycelium with light petroleum. Such extracts normally contain steroidal material, e.g. ergosterol (Thomas, 1955).

With [2-^{14}C]mevalonic acid lactone the count rate was approximately 16-fold in excess of the background count, thus demonstrating significant incorporation. On the other hand, [1-^{14}C]mevalonic acid lactone, which would not be expected to produce appreciable labelling in normal steroidal components because of the decarboxylation step accompanying condensation of these units, in fact gave rise to only comparatively slight activity in the lipoid fraction (Table 1). It follows from these findings that although mevalonic acid is metabolized by *P. cyclopium*, it is apparently not a normal intermediate in the biosynthesis of palitantin.

EXPERIMENTAL

All melting points are uncorrected.

Culture and medium. The culture used to obtain palitantin was a strain of *Penicillium cyclopium* Westling (London School of Hygiene Catalogue no. 525) grown on Czapek-Dox medium [cf. Bracken, Pocker & Raistrick (1954)].

Radioactive assay. All measurements were carried out with a Panax scaler (type 100C) coupled to an end-window Geiger–Müller tube and compared with a polymethyl methacrylate standard of known ^{14}C content (1 μc/g.).

Isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. Samples were counted at infinite thickness with planchets 1·5 cm. in diameter, the standard error not exceeding $\pm 3\%$. All the activities quoted in Tables 1 and 2 are corrected for background (approx. 9 counts/min.).

Determination of palitantin production. Ninety-six 1 l. flasks containing Czapek-Dox medium (350 ml./flask) were inoculated with a suspension of *P. cyclopium* in sterile water and incubated in the dark at 24°. At regular intervals batches of eight flasks were selected at random and examined for pH and glucose content (determined polarimetrically). The culture filtrate was extracted with ether

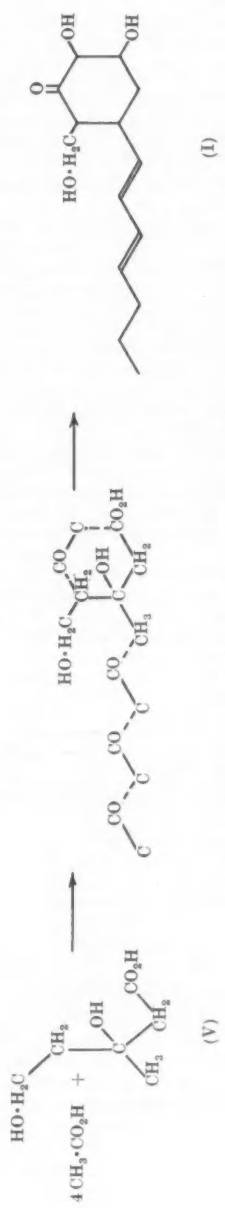

Fig. 4. Suggested incorporation of mevalonic acid into palitantin.

(4 × 0·25 vol.) which was then partially evaporated before shaking with 2 N-Na_2CO_3 and subsequent separation into carbonate-soluble (A) and carbonate-insoluble (B) fractions. Fraction (A) yielded crude frequentin accompanied by a yellow syrupy contaminant; concentration of fraction (B) yielded palitantin as a crude crystalline deposit. The results are shown graphically in Fig. 2.

Incorporation of [1-^{14}C]*acetate.* *P. cyclopium* was cultured as before and after 8 days 40 μc of sodium [1-^{14}C]acetate in sterile water (5 ml.) was added to each of three flasks. These were harvested at 11, 13 and 15 days and examined as described above. In addition the mycelium was washed, dried and Soxhlet-extracted, initially with light petroleum (b.p. 40–60°) for 18 hr. and subsequently with ether for 30 hr. The activities of the crude palitantin and frequentin fractions and of the crude mycelial extracts were determined (Table 1).

Purification and dilution of labelled palitantin. The combined palitantin fractions of the 11-, 13- and 15-day flasks were purified by three sublimations in high vacuum at 130–135°, giving colourless crystals of palitantin (208 mg.) of constant m.p. (164–165°) and radioactivity (6370 counts/min., 24 μc/g.). This sample was mixed with pure inactive palitantin (1·792 g.) and dissolved in warm acetone (30 ml.), which was then evaporated to yield diluted palitantin (2 g.) with activity, 641 counts/min. (unchanged on vacuum sublimation).

Degradation of labelled palitantin

Kuhn–Roth oxidation. A Kuhn–Roth oxidation of palitantin (0·508 g.) (cf. Birkinshaw, 1952), gave a quantitative yield of acetic acid which was separated by steam-distillation and neutralized with 0·1 N-LiOH. The dried lithium acetate was pyrolysed in a current of N_2 at 390° for 10 min. and the effluent acetone was absorbed in water and converted into iodoform by the addition of aqueous alkaline iodine. The resulting iodoform was inactive.

The Li_2CO_3, remaining after pyrolysis was acidified with lactic acid, heated and the resulting CO_2 was passed, in a stream of N_2, into 0·2 N-$Ba(OH)_2$, giving active $BaCO_3$ which was then assayed.

Ozonolysis. Ozonized oxygen was passed through an ice-cooled solution of palitantin (0·508 g.) in aldehyde-free $CHCl_3$ (75 ml.) for 3 hr. The solution was extracted [by shaking with an equal volume of water and, after separation, the aqueous phase was heated to 60°]. The effluent *n*-butyraldehyde was passed in a current of N_2 through bubblers containing Brady's reagent (2:4-dinitrophenylhydrazine in 2 N-HCl). The deposited phenylhydrazone (0·131 g., m.p. 121–123°) was purified by recrystallization from aqueous 50% ethanol, giving lemon-yellow needles, m.p. 123°, which were then assayed directly.

Periodic acid oxidation. Palitantin (0·254 g.) in ethanol (25 ml.) was treated with a solution of potassium periodate (0·508 g.) in CO_2-free water (100 ml.). After 72 hr. mercuric oxide (3 g.) was added and the solution was refluxed in a stream of N_2 for 2 hr. in order to convert the formic acid formed initially into CO_2 (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The CO_2 was precipitated as $BaCO_3$ (yield 91% of the theoretical), which on assay proved to be inactive.

The periodate oxidation of palitantin was repeated but this time, after 72 hr. the solution was concentrated by distillation. The distillate was treated with excess of lead

carbonate, heated and filtered. The filtrate was then concentrated and cooled, when a precipitate (25 mg.), apparently identical with lead formate, separated. This also had zero activity.

The values of the radioactivities of the fragments obtained in the above degradations are summarized in Table 2.

Attempted incorporation of labelled mevalonic acid lactone

DL-[1-^{14}C]Mevalonic acid lactone (25 μc) was added in sterile water to a flask containing an 8-day culture of *P. cyclopium*. A second flask was similarly treated with DL-[2-^{14}C]mevalonic acid lactone (10 μc). After a further 3 days the flasks were harvested and the palitantin was extracted as before but in neither case did it show any significant activity. In addition the mycelium was Soxhlet-extracted with light petroleum (b.p. 40–60°) for 18 hr. and the extract was concentrated to dryness. On assay the crude product from [2-^{14}C]mevalonic acid lactone was significantly active (147 counts/min.), whereas the product from the [1-^{14}C]-lactone was only slightly active (26 counts/min.), cf. Table 1. The lipoid fraction was assumed to contain steroidal material, e.g. ergosterol (Thomas, 1955).

SUMMARY

1. The biosynthesis of palitantin, $C_{14}H_{22}O_4$, an alicyclic metabolite of *Penicillium palitans* and also of *Penicillium cyclopium*, has been studied.

2. Degradations of labelled palitantin derived from [1-^{14}C]acetate strongly support a biosynthetic mechanism involving the head-to-tail condensation of acetate units.

3. The biosynthetic pathway does not appear to involve mevalonic acid.

We are grateful to Imperial Chemical Industries Ltd. for a grant towards the purchase of tracer equipment and ^{14}C-labelled compounds. One of us (R.T.) participated in this work during the tenure of a Wellcome Research Fellowship. We wish to thank Professor J. H. Birkinshaw for much helpful discussion during the course of this work.

REFERENCES

Birch, A. J., Fitton, P., Pride, E., Ryan, A. J., Smith, H. & Whalley, W. B. (1958). *J. chem. Soc.* p. 4576.

Birch, A. J., Schofield, J. & Smith, H. (1958). *Chem. & Ind.* p. 1321.

Birkinshaw, J. H. (1952). *Biochem. J.* **51**, 271.

Birkinshaw, J. H. & Raistrick, H. (1936). *Biochem. J.* **30**, 801.

Bowden, K., Lythgoe, B. & Marsden, D. J. S. (1959). *J. chem. Soc.* p. 1662.

Bracken, A., Pocker, A. & Raistrick, H. (1954). *Biochem. J.* **57**, 587.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. E. (1949). *Isotopic Carbon*, p. 258. New York: John Wiley and Sons Inc. London: Chapman and Hall Ltd.

Gould, B. S. & Raistrick, H. (1934). *Biochem. J.* **28**, 1640.

Quilico, A., Cardani, C. & Lucattelli, I. (1953). *Gazz. chim. ital.* **83**, 1088.

Thomas, R. (1955). *Aust. J. Chem.* **8**, 142.

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A Further Genetic Variety of Glutamic Acid Dehydrogenase in *Neurospora crassa*

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The amination-deficient mutants of *Neurospora crassa* all appear to be deficient in glutamic dehydrogenase, and are all due to genetic change within a short segment (the *am* locus) of the chromosome corresponding to linkage group 5 (Pateman & Fincham, 1958; Fincham, 1959*a*; R. W. Barratt, personal communication). Treatment of conidia of several of these mutants with ultraviolet light has resulted in the induction of apparent reversions to the wild-type condition. Some of the 'revertants' were indistinguishable from the standard, wild-type organism, whereas in others there seemed to have been only partial repair of the original mutational damage to the genetic basis of glutamic-dehydrogenase formation (Pateman, 1957). Two of these imperfect revertants have now been investigated in detail. The first, derived from the enzyme-negative allele *am*^{2} [the revised gene symbols adopted in Fincham (1959*a*) are used here], and designated *am*^{21}, has been shown to produce an abnormal kind of glutamic dehydrogenase which is almost inactive as normally extracted, but

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can be activated by mild heat treatment (Fincham, 1957). The second revertant is described in this paper and is shown to produce another variety of the enzyme, the abnormality in this case consisting, most conspicuously, of increased Michaelis constants for all substrates.

MATERIALS AND METHODS

Origins of strains of Neurospora. The standard wild-type organism, used throughout, was either St Lawrence *A* (ST*A*, sometimes referred to in the literature as 74*A*) or 2*a*, a strain of the opposite mating type closely related to ST*A* through inbreeding. The original partial revertant was obtained by Pateman (1957) and was given the number 1038. It originated through ultraviolet treatment of a strain of mating-type *a* which was devoid of glutamic-dehydrogenase activity because of the presence of the allele am^3. Evidence, to be considered below, indicates that 1038 and its descendants carry a new allele, derived from am^3 and designated am^{3a}. The strain 26–7 (*A* am^{3a}) was derived from the cross 1038 × ST*A*, whereas 34–1 (*a* am^{3a} *albino*) came from 26–7 × an albino strain of mating-type *a*, and 1566–78 (*a* am^{3a}) from 34–1 × ST*A*. B317 and 46004 are unlinked arginine-requiring mutants, the first lacking argininosuccinase and the second probably deficient in the enzyme which forms argininosuccinate (Newmeyer, 1957). The double mutant 42–4 (*a* am^{3a} *arg*-10) was isolated from 26–7 × B317 (*a arg*-10).

Reagents. α-Oxoglutaric acid and glutamic acid were obtained from Roche Products Ltd., and were used as the sodium salts. Triphosphopyridine nucleotide (TPN) of 95–100% purity was obtained from the Sigma Chemical Co. through G. T. Gurr Ltd., 136, New Kings Road, London, S.W. 6. Reduced TPN (TPNH) was prepared in solution by the general method of Evans & Nason (1953), with a slight excess of DL-*iso*citrate and an ammonium sulphate (50–60% saturation) fraction of pig-heart extract, dialysed before use, as source of *iso*citric dehydrogenase.

Measurement of enzyme activity. Enzyme activity was measured spectrophotometrically essentially as described by Fincham (1957), the temperature being controlled by circulating water. Reaction mixtures were buffered with 0·08–0·09 M-orthophosphate at pH 8·0; glass-electrode measurements confirmed that this buffer was effective in maintaining the pH at this value. Normally a Na_2HPO_4–NaH_2PO_4 (94:6) mixture was used, but where high levels of NH_4^+ ion were required it was partly replaced by $(NH_4)_2HPO_4$ at pH 8·0, the phosphate concentration being kept constant. The final component of the reaction mixture (usually enzyme, but sometimes ammonium salt) was added in 0·1 ml., from a depression in a Perspex plunger, which was used to obtain virtually instantaneous mixing of the reactants. The unit of activity is an initial rate of change of $E_{340\,m\mu}$ of 0·001/min. Specific activity is expressed as units/mg. of protein and, unless otherwise stated, refers to a system which is 6·7 mM-α-oxoglutarate, 13·3 mM-NH_4Cl and approx. 60 μM-TPNH.

Enzyme preparations. Mycelium was grown, from heavy inocula of conidia, for 48 hr. at 25° without agitation. Small quantities of mycelium for the preparation of crude extracts were grown in 50 ml. of medium in 250 ml. conical flasks, and larger quantities for enzyme purifications were grown in 100 ml. of medium in Roux bottles. The medium was the liquid minimal medium 'N' of Vogel & Bonner (1956). Moist mycelial pads, after being washed with water and blotted with paper towelling, were ground in a chilled mortar with powdered glass and about five times their weight of 0·05 M-phosphate, pH 8·0. Further buffer was added, after grinding, to give a total of 10–30 times the original weight of moist mycelium, and the homogenate was filtered through a washed pad of kieselguhr on a Büchner funnel. The specific activity of the filtrate depended on the amount of kieselguhr used in relation to the amount of protein in the extract. For small quantities of mycelium, where no further purification was intended, 2 g. of kieselguhr was used for each 50 ml. of culture (about 0·5 g. of mycelium). The filtrates then had specific activities of 1000–1500 for the wild type. Where larger quantities were being processed for enzyme purification, about 1 g. of kieselguhr was used/g. of moist mycelium, and the filtrates then had specific activities, for the wild type, of only about 500, owing to the higher recovery of protein. Further purification was carried out by $(NH_4)_2SO_4$ fractionation, followed by adsorption on to, and elution from, calcium phosphate gel, according to the procedure described by Fincham (1959*b*). The final preparations were purified at least 40-fold as compared with the kieselguhr filtrate, and had specific activities, for the wild type, of 20 000–30 000 or slightly more. Preparations of this type will be referred to as '40-fold purified enzyme'. The recovery of activity tended to vary according to the particular batch of gel being used; the most successful batch gave about 80% recovery of activity in the combined fractions, with about 25% of the original activity in the most active gel eluate. The glutamic dehydrogenase from am^{3a} strains appeared to have the same fractionation characteristics as the wild-type enzyme. In a few experiments, $(NH_4)_2SO_4$ fractions were used without gel treatment (e.g. Table 2).

Protein determinations. Protein was determined colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951), with reference to a standard curve prepared with casein.

RESULTS

Distinguishing properties of wild-type, 2l and 3a enzymes

In Fig. 1 are shown the results of tests which show that the glutamic dehydrogenase produced by am^{3a} strains (3a enzyme) behaves differently both from wild-type enzyme, and from the enzyme type characteristic of am^{2l} strains (2l enzyme).

Unlike the wild-type enzyme, both the 2l and the 3a enzymes can, in crude kieselguhr filtrates, be activated by a few minutes of mild heat treatment (38° was the temperature generally used in the present experiments). In both cases partial activation can also be achieved as a result of incubation with substrate mixtures. However, whereas with the 2l enzyme both TPNH and α-oxoglutarate must be present before any activation occurs (Fincham, 1957), the 3a enzyme is considerably activated by incubation in reaction mixtures

minus TPNH [cf. curves 3 and 5, Fig. 1 (*b*) and (*c*)]. The 3a enzyme resembles the 2l enzyme in showing no further activation by substrates after it has been activated by heat [Fig. 1 (*b*), curves 4 and 6]. When the 3a enzyme is incubated for 3 min. in the reaction mixture minus TPNH before the start of the reaction, a prior heat-activation of the enzyme increases the activity only by about 50 % [curves 5 and 6, Fig. 1 (*b*)]. The 2l enzyme, however, shows at least a tenfold increase in initial reaction rate as a result of heat-activation under these conditions [curves 5 and 6, Fig. 1 (*c*)]. Wild-type enzyme typically shows no response to any of these treatments and appears to be already fully active as extracted from the mycelium.

Decrease in NH_4^+-ion concentration from 0·1 M to 3·3 mM causes a decrease in reaction rate catalysed by the 3a enzyme by a factor of 8–9 [curves 2 and 4, Fig. 1 (*b*)], whereas the corresponding decrease for both the wild-type and 2l enzymes is no more than 25 %.

The data shown in Fig. 1 indicate that, whereas the am^{2l} extract when fully activated still had less than half the specific activity of the wild-type extract, the maximum activity (at high NH_4^+-ion concentrations) of the am^{3a} extract was actually considerably higher than the wild-type value. These results are quite typical of several experiments other than the one shown.

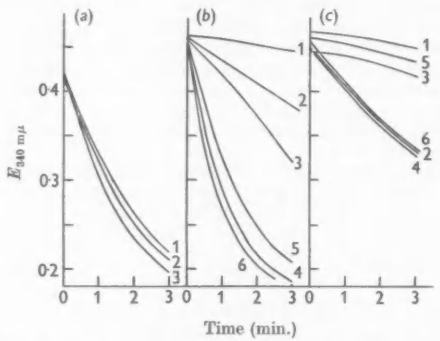

Fig. 1. Enzyme assays showing the different properties of wild, 3a- and 2l-type glutamic dehydrogenases. Kieselguhr-filtered extracts of the strains ST*A* (am^+) (*a*), 1566–78 (am^{3a}) (*b*) and 1361–12 (am^{2l}) (*c*) were used, the amounts of protein per assay being 85, 79 and 90 μg. respectively. Reaction mixtures contained, in 3·0 ml., 20 μmoles of α-oxoglutarate and 0·18 μmole of TPNH. Temp., 21°. Numbers on the curves indicate the following conditions: 1 and 2, 10 μmoles of NH_4^+ ions; 3–6, 300 μmoles of NH_4^+ ions; 2 and 4, enzyme warmed to 38° for 5 min. and cooled rapidly to 21° immediately before adding to the complete reaction mixture; 5, enzyme incubated for 5 min. in the reaction mixture minus TPNH, and the TPNH added (in 0·2 ml.) at zero time; 6, enzyme heat-treated as in 2 and 4, and then incubated with the substrate mixture as in 5. In this experiment procedures 4, 5 and 6 were omitted for the wild-type enzyme, other experiments having shown 3–6 to give identical results with the wild type.

The three enzyme types can also be quite sharply distinguished on the basis of thermostability. In one experiment in which crude kieselguhr filtrates were compared directly, 3a enzyme lost about 35 % of its activity during 3 min. at 60·5° (in phosphate buffer at pH 8·0), whereas 2l enzyme was at least 98 % inactivated under the same conditions. Similar preparations from wild type are typically almost completely stable at this temperature.

In the following sections the properties of the 3a enzyme will be described in more detail.

Conditions for activation of the 3a *enzyme*

The activation behaviour of crude 3a filtrates has been described in the preceding section. Preparations of 3a enzyme purified 40-fold have given rather variable results. Two such preparations, one from strain 26–7 and one from strain 1566-78, behaved as if already fully activated in that they gave no increase in reaction rate as a result of incubation with substrates. Other preparations, however, showed pronounced activation effects (Table 1). Although a certain amount of activation occurred as a result of incubation in reaction mixture minus α-oxoglutarate, the presence of this substrate is necessary for the maximum effect. In another experiment, α-oxoglutarate in phosphate buffer, in the absence of both TPNH and NH_4^+ ion, could activate this enzyme preparation.

Purified preparations, which were activated by exposure to substrates, were activated even more by warming to 38° for a few minutes. After heat-activation no further activation could be achieved as a result of incubation with substrates. Unlike the activated form of the 2l enzyme, which has a half-life of about 30 min. at 21° (Fincham, 1957, 1960), the activated form of the 3a enzyme

Table 1. *Effects of incubation with substrate mixtures on the activity of a preparation of enzyme* 3a

Mixtures at approx. 20° contained 0·8 M-sodium phosphate, pH 8·0, 6·7 mM-α-oxoglutarate, 3·3 mM-ammonium chloride, approx. 45 μM-TPNH and 60 μg. of 40-fold purified enzyme from strain 26–7. All the components except one were mixed in 2·8 or 2·9 ml., and the last component was added 3 min. later to bring the volume to 3·0 ml.

Component added last	NH_4^+ ions	α-Oxoglutarate	TPNH	Enzyme
Initial rate of reaction ($10^3\ \Delta E_{340\ m\mu}$/min.)	78	39	75	13

Table 2. *Thermostabilities of wild-type and* 3a*-type enzyme preparations separately and in mixture*

Reaction mixtures contained 6·7 mM-α-oxoglutarate, 3·3 mM-ammonium chloride, approx. 55 μM-TPNH and 0·12 mg. of protein from *am*^{3a} (26–7), or 0·05 mg. of protein from wild type, or both. The fractions used were precipitated from crude extracts with 45% saturated ammonium sulphate (added as the solid salt). Before testing, enzyme samples were heated at 58° for various times in a water bath. 'Zero' time of heating was taken as 1 min. after placing in the bath, corresponding approximately to the time at which temperature equilibrium was attained.

	Wild type			Type 3a			Mixture		
Time of heating (min.)	0	3	9	0	3	9	0	3	9
Activity (units/min.; initial rate)	40	40	40	28	7	2	70	48	42
Theoretical for no interaction	—	—	—	—	—	—	68	47	42

appeared to be quite stable at room temperature. In one experiment with a 40-fold-purified preparation from strain 1566–78, almost no activity was lost during 2 hr. at 21° after activation at 38°.

Similarly purified wild-type preparations normally show no activation, either through warming or through incubation with substrates at pH 8·0, though activation as a result of incubation with an α-oxoglutarate–phosphate mixture has been observed with one rather old and partially inactivated wild-type preparation.

Thermostability

As already mentioned, glutamic dehydrogenase of type 3a is considerably more thermolabile than the wild-type enzyme, though less so than the 2l type. Thermostability varies with the stage of purification (more purified preparations being less stable), and probably also with other factors which have not been systematically investigated, such as protein concentration and the volume of the solution heated. Consequently it is not possible to give generally meaningful values for half-lives at different temperatures. Nevertheless, comparable preparations of wild-type and 3a-type enzymes have always shown large differences in thermostability when tested under the same conditions.

In order to confirm that these differences were due to an intrinsic difference between two kinds of enzyme molecule, rather than to other factors such as protein concentration or the presence in one or other type of preparation of stabilizing or destabilizing substances, a heat-inactivation experiment was performed in which wild-type enzyme and 3a-enzyme preparations were heated separately and in a mixture. The results (Table 2) showed that the stability of each kind of glutamic dehydrogenase was unaffected by mixture with the other kind of preparation.

Michaelis constants

Michaelis constants for all five substrates were determined for 40-fold-purified preparations from both wild type and strain 1566–78 (*am*^{3a}). The preparations of the 3a enzyme were heated to 38° for 4 min. immediately before addition to reaction mixtures in order to exclude activating effects of substrates.

Figs. 2–4 show that the Michaelis constant for glutamate (K_{GA}) and that for TPN (K_{TPN}) are both very much higher for 3a enzyme than for wild-type enzyme. Fig. 2 shows, in agreement with the finding of Frieden (1959*c*) for ox-liver glutamic dehydrogenase, that with the wild-type *Neurospora* enzyme the apparent Michaelis constant for each of these two substrates decreases as the concentration of the other is increased. The constants estimated from the data of Fig. 2 for wild-type enzyme are: 7·0 mM for K_{GA}, 26 μM for K_{TPN} and $1{\cdot}1 \times 10^{-6}$ M^2 for the complex constant $K_{GA.TPN}$ (Alberty, 1953). For the 3a enzyme, both K_{GA} and K_{TPN} are so high that it has not been possible to determine the Michaelis constant of either substrate in the presence of nearly saturating concentrations of the other. Consequently, the values obtained from the data in Figs. 3 and 4 (0·1 M for K_{GA} and 0·4 mM for K_{TPN}) may be over-estimates. However, the fact that nearly the same estimate of K_{GA} was obtained at two rather widely different TPN concentrations (Fig. 3) suggests that any such over-estimation was not very great and that, for 3a enzyme, $K_{GA.TPN}$ is approximately equal to $K_{GA} \cdot K_{TPN}$.

The 3a enzyme also had abnormally high Michaelis constants for all three substrates involved in the reverse reaction (glutamate formation). The wild-type Michaelis constant for α-oxoglutarate (K_{OGA}) has been determined, both during the present work and by Fincham (1957, 1960*a*), and is about 3·5 mM, and that for NH_4^+ ion ($K_{NH_4^+}$) is between 1·0 and 1·5 mM. The wild-type Michaelis constant for TPNH (K_{TPNH}) appeared, from repeated experiments (e.g. Fig. 5), to be close to 16 μM. This is a somewhat lower value than that reported by Fincham (1952*b*), the difference being probably due to a tendency in the earlier work to under-estimate initial rates of reaction at lower levels of TPNH. Each of these Michaelis constants was determined at nearly saturating concentrations of the non-varying substrates. For the 3a enzyme, however, the relatively high Michaelis

constants made saturation of the enzyme with α-oxoglutarate and TPNH impossible to achieve without making $E_{340\ m\mu}$ undesirably high. Frieden (1959*c*) found that, for the ox-liver enzyme, the apparent Michaelis constant for each of the substrates α-oxoglutarate and TPNH increased with the concentration of the other, provided that the concentration of NH_4^+ ion was high. This effect has not, however, been found for the *Neurospora* 3a enzyme, and the indications are that the constant for each substrate is nearly independent of the concentrations of the other two. Thus Fig. 5 shows nearly the same estimate of K_{TPNH} obtained at quite widely different α-oxoglutarate and NH_4^+-ion concentrations, and Fig. 6 shows nearly the same estimate of K_{OGA} obtained at two different concentrations of ammonium salt. It thus seems likely that the estimates of Michaelis constants indicated by Figs. 5–7, and tabulated in Table 3, are not far from the true values. K_{OGA} and K_{TPNH} are each about three times and $K_{NH_4^+}$ about 30 times as high for 3a enzyme as for wild-type enzyme. The large difference in respect of $K_{NH_4^+}$ is also evident in the results of the experiments on crude extracts shown in Fig. 1.

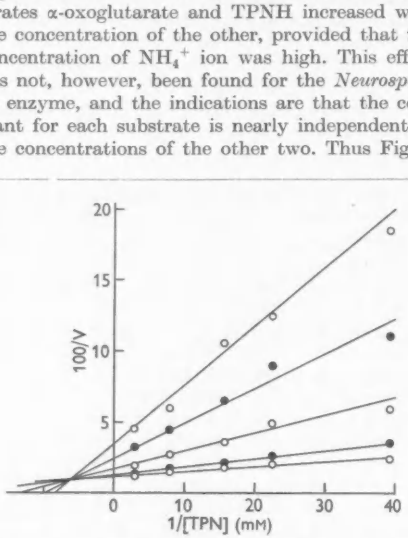

Fig. 2. Relationship between glutamate and TPN concentrations, and velocity of the forward reaction catalysed by 40-fold-purified wild-type enzyme. Reaction mixtures at 25·5° each contained 8 μg. of protein from wild type (STA) in 3·0 ml. Lines fitted to the experimental points are for the following concentrations of glutamate, reading from top to bottom: 3, 6, 15, 40 and 100 mM.

The very strikingly distinct values of $K_{NH_4^+}$ provided a good opportunity for an additional check of the hypothesis of an intrinsic difference between the wild-type enzyme and 3a enzyme. The results of an experiment on mixed 3a and wild-type preparations (Fig. 7) show that each type of enzyme retains its own characteristic affinity for NH_4^+ ion (or ammonia) in the presence of all the components of the other type of enzyme preparation.

Comparative maximum velocities

Comparison of 40-fold-purified 3a and wild-type glutamic-dehydrogenase preparations showed that the ratio of the rate of the back reaction (glutamate synthesis) to that of the forward reaction was very much lower with the wild-type enzyme than with the 3a enzyme. The comparison, at relatively high concentrations of all substrates, is shown in Table 4. At these substrate concentrations the reaction velocities obtained with the wild-type enzyme must

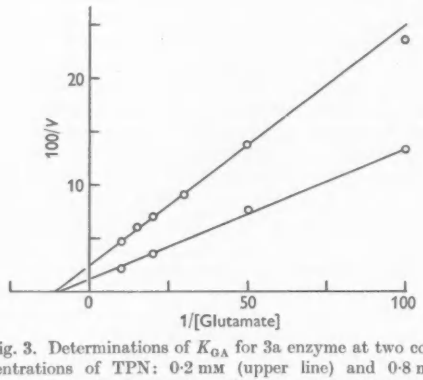

Fig. 3. Determinations of K_{GA} for 3a enzyme at two concentrations of TPN: 0·2 mM (upper line) and 0·8 mM (lower line). Each reaction mixture contained 30 μg. of protein of 40-fold-purified 3a enzyme. Temp., 21°. The indicated Michaelis constant is approx. 0·1 M at both TPN concentrations.

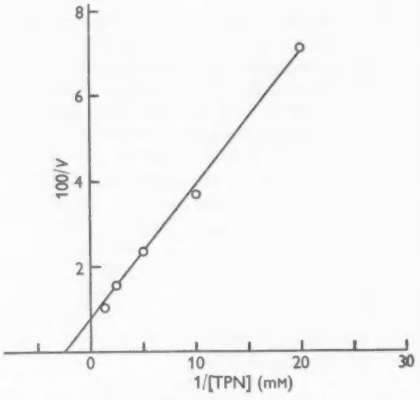

Fig. 4. Determination of K_{TPN} for 3a enzyme. Each reaction mixture contained 60 μg. of protein of 40-fold-purified 3a enzyme, with a glutamate concentration of 0·1 M. Temp., 21°. The indicated Michaelis constant is 0·4 mM.

have been fairly close to maximal in both reaction directions. For the 3a enzyme, however, and on the basis of the Michaelis constant listed in Table 3, the observed forward and backward velocities should be multiplied by 3·0 and 2·6 respectively to give maximal velocities. Thus the ratio $V_{max.\ (back)}/V_{max\ (forward)}$ is about 5 for wild-type and about 45 for 3a-type enzyme.

This difference is quite consistent with the differences in Michaelis constants already noted. According to Frieden (1959*c*), the equilibrium constant for the reaction system should be obtainable from the expression

$$K = \frac{V_{max.\ (forward)}}{V_{max.\ (back)}} \cdot \frac{K_{TPNH.OGA.NH_4^+}}{K_{TPN.GA}} \cdot [H^+].$$

Taking the value of the complex constant $K_{TPN.GA}$ for wild-type enzyme as that given by the data of Fig. 2, and making the assumptions (which are at least consistent with the data of Figs. 3, 5 and 6) that, for 3a enzyme, $K_{TPN.GA}$ is approximately equal to $K_{TPN} \cdot K_{GA}$, and that, for both enzyme types, the complex constant $K_{TPNH.OGA.NH_4^+}$ is approximately equal to $K_{TPNH} \cdot K_{OGA} \cdot K_{NH_4^+}$, the equilibrium constant at 21° is calculated to be $1{\cdot}0 \times 10^{-13}$ M² from the 3a data and $1{\cdot}2 \times 10^{-13}$ M² from the wild-type data. The agreement with the value of $1{\cdot}0 \times 10^{-13}$ M² determined from the equilibrium measurements of Olsen & Anfinsen (1953) (which were, however, made at 25°) is closer than could have been expected, in view of the various uncertainties involved in the calculations.

The maximum specific activity for wild-type enzyme in the forward reaction is greater than that measured for crystalline ox-liver glutamic dehydrogenase at a slightly less favourable pH (7·6), but otherwise under very similar conditions (Strecker, 1955). The wild-type enzyme preparation used in the experiment recorded in Table 4 had been stored frozen at −10° for 6 weeks, and was more active when freshly made.

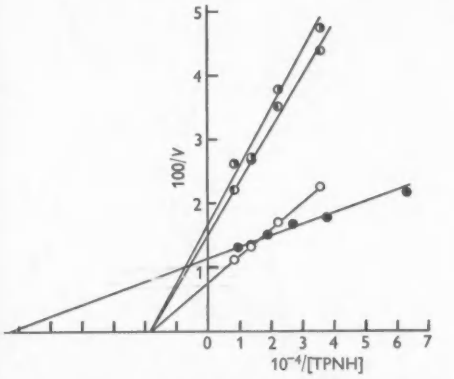

Fig. 5. Determinations of Michaelis constants for TPNH for 3a and wild-type enzymes. In the experiment with 3a enzyme, 1·6 μg. of a 40-fold-purified preparation was present in each reaction mixture, with the following concentrations of NH_4^+ ion and α-oxoglutarate respectively: 97 and 19·4 mM (○); 19·4 and 19·4 mM (◐); 97 and 3·9 mM (◑). In the experiment with wild-type enzyme (●), 1·2 μg. of protein of a 40-fold-purified preparation was present in each reaction mixture, with 26·7 mM-NH_4^+ ion and 16·7 mM-α-oxoglutarate. Temp., 21°. The indicated values of K_{TPNH} are 16 μM for wild-type and 56 μM for 3a enzyme.

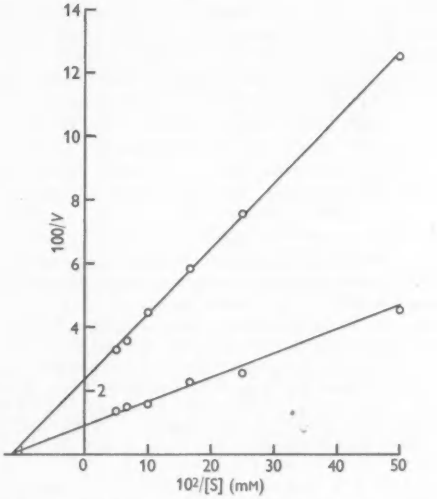

Fig. 6. Determinations of Michaelis constant for α-oxoglutarate for 3a enzyme at two concentrations of NH_4^+ ion [added as $(NH_4)_2HPO_4$]: upper line, 0·02 M; lower line, 0·1 M. The TPNH concentration was 77 μM; 2 μg. of 40-fold-purified enzyme was present in each reaction mixture. Temp., 21°.

Table 3. *Michaelis constants for* 3a *and wild-type glutamic dehydrogenases*

Enzyme type	Substrate: L-Glutamate (mM)	α-Oxoglutarate (mM)	NH_4^+ ion (mM)	TPN (μM)	TPNH (μM)
Wild	7	3·5	1·2	26	16
3a	100	9	35	400	56

Characteristics of the glutamic dehydrogenase formed in $am^+ + am^{3a}$ *heterocaryons*

One might expect a heterocaryon containing a mixture of am^+ and am^{3a} nuclei in a common cytoplasm to produce a mixture of the two kinds of enzyme characteristic of the respective homocaryons. To check this point, a forced heterocaryon was made from strain 42–4 (am^{3a} *arg*-10) and strain 46004 (am^+ *arg*-1). Growth on minimal-agar medium, resulting from simultaneous inoculation of these two strains, was necessarily heterocaryotic, since neither homocaryon is capable of growing in the absence of arginine. The results of an experiment on an extract of this heterocaryon, grown in liquid minimal medium, are shown in Fig. 8. The relation between reaction velocity and NH_4^+-ion concentration was of the same type as obtained with an artificial mixture of wild-type and 3a-type preparations (cf. Fig.7). A mixture of wild-type and 3a enzymes in the proportion 1:3·4 (in terms of their respective maximum velocities) would account for the heterocaryon data. When the extract was heated to 60·5° for 30 min. (a treatment known to destroy most of the activity of am^{3a} extracts and scarcely affecting wild-type activity), the effect was such as would be expected if 70% of the 3a-enzyme component, but none of the wild-type component, had been destroyed. Thus the results are in accordance with the

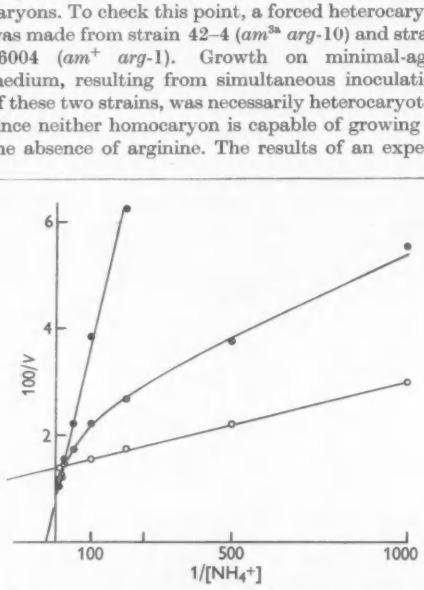

Fig. 7. Plots of reciprocal velocities versus reciprocal NH_4^+ ion concentration for: ●, 3 μg. of 40-fold-purified 3a enzyme (from strain 1566–78); ○, 2·5 μg. of 40-fold-purified wild-type enzyme (from ST*A*); (⊘) 1·5 μg. of the 3a enzyme mixed with 1·25 μg. of the wild-type enzyme, each in 3·0 ml. Concentrations of the other reactants were: α-oxoglutarate, 6·67 mM; TPNH, approx. 65 μM. Temp., 21°. Lines drawn are theoretical for $K_{NH_4^+}$ 1·18 mM and $V_{max.}$ 72·5 for the wild-type enzyme, $K_{NH_4^+}$ 35 mM and $V_{max.}$ 125 for the 3a enzyme, and no interaction in the mixture.

Table 4. *Forward and back reaction velocities catalysed by wild-type and* 3a *enzyme preparations* (40-*fold purified*)

Reaction mixture I contained: 0·12 M-NH_4^+ ion [as $(NH_4)_2HPO_4$]; 0·02 M-α-oxoglutarate; 0·15 mM-TPNH; 0·07 M-orthophosphate. Reaction mixture II contained: 0·1 M-L-glutamate; 0·85 mM-TPN; 0·08 M-orthophosphate. Both were at pH 8·0.

Source of enzyme	Specific activities ($\Delta E_{340\ m\mu}$/min./mg. of protein) Mixture I	Mixture II	Ratio of velocities (back/forward)
ST*A* (am^+)	32 000	6520	4·9
1566–78 (am^{3a})	62 000	1220	51

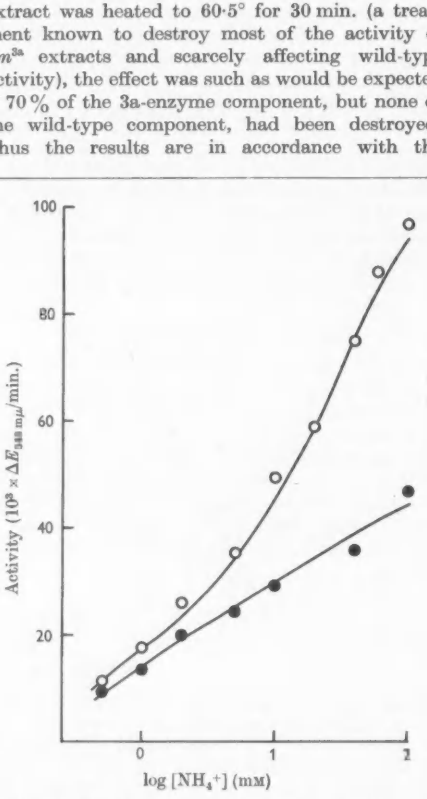

Fig. 8. Relation between NH_4^+ ion concentration and velocity of reaction catalysed by a kieselguhr-filtered extract of the heterocaryon 42–4 (am^{3a} *arg*-10) + 46004 (am^+ *arg*-1). ○, Extract unheated (except for preliminary activation at 38°); ●, extract heated at 60·5° for 30 min. before testing. Concentrations of the other reactants were: α-oxoglutarate, 6·67 mM; TPNH, approx. 65 μM; approx. 35 μg. of extract protein was present in each reaction mixture. Temp., 21°. The curves drawn are theoretical on the assumptions that the unheated extract contained a mixture of wild-type enzyme, with $K_{NH_4^+}$ 1·2 mM and $V_{max.}$ 27, and 3a enzyme, with $K_{NH_4^+}$ 35 mM and $V_{max.}$ 92; and that the 3a component was 72% inactivated by the heat treatment whereas the wild-type component was unaffected.

expectation that an $am^+ + am^{3a}$ heterocaryon will produce two types of glutamic dehydrogenase, one with a high affinity for NH_4^+ ion and high thermostability and one with much lower affinity for NH_4^+ ion and lower thermostability.

Inheritance of am^{3a}

The evidence that the formation of the distinct 3a type of enzyme is due to a new allele (am^{3a}) at the *am* locus is derived principally from the analysis of crosses between wild and 3a-type strains. Cultures grown from ascospores from such crosses were scored by determination of their glutamic-dehydrogenase type, since no reliable method has been devised for distinguishing 3a types on the basis of growth characteristics. Two criteria were used for distinguishing the two enzyme types in crude kieselguhr filtrates: the relatively low activity shown by the 3a extracts at low concentrations of NH_4^+ ion and the relatively low thermostability of this activity. The two criteria were always found to agree (Fig. 9).

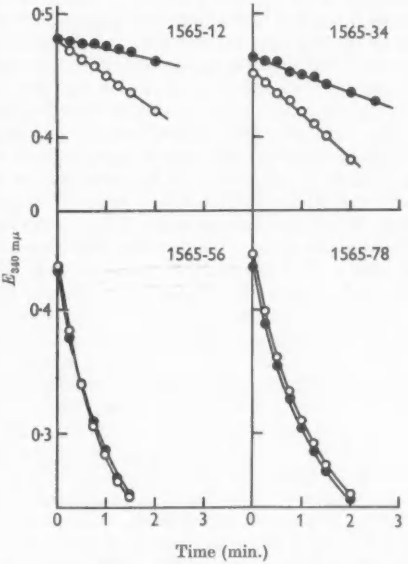

Fig. 9. Segregation of am^+ from am^{3a} in an ascus from the cross ST*A* (am^+) × 34–1 (am^{3a}). The curves represent glutamic-dehydrogenase assays on the kieselguhr-filtered extracts of cultures grown from the four spore pairs of ascus no. 1565. The assay system contained, in 3·0 ml., 20 μmoles of α-oxoglutarate, 40 μmoles of ammonium chloride (added to start the reaction), approx. 0·15 μmole of TPNH and 0·2 ml. of mycelial extract containing 0·30, 0·28, 0·18 and 0·18 mg. of protein in 1565-12, -34, -56 and -78 respectively. ○, Extracts unheated; ●, extracts heated to 61·5° for 15 min. immediately before assay.

Seventeen asci from crosses between 3a strains and wild type (six from 1038 × ST*A*, five from 26–7 × *albino a*, four from 26–7 × B 317 and two from 34–1 × ST*A*) were each dissected into the four spore pairs; the ascospores were germinated and the resulting cultures were scored for glutamic-dehydrogenase type. A typical result is shown in Fig. 9. In each case, two of the spore pairs gave cultures producing enzyme of the 3a type whereas the other two yielded wild-type enzyme. In addition, strain 1038 was crossed to a strain of mating-type *A*, which carried the 'enzyme-negative' allele am^1. Four asci from this cross each contained two spore pairs which gave cultures of the typical 3a type, and two which gave cultures apparently devoid of any glutamic-dehydrogenase activity.

These results are consistent with the hypothesis that the 3a-type enzyme is determined by a new allele at the *am* locus. They do not, however, rule out the alternative possibility of the 3a phenotype's being due to the unchanged am^3 allele together with a 'suppressor' mutation in a distinct, though linked locus. Unless such a suppressor was extremely closely linked to *am*, one would expect enzyme-negative am^3 ascospores to be produced as a result of genetic recombination in 3a × wild-type crosses, if the suppressor hypothesis were true. Such ascospores can be distinguished both from 3a type and from wild-type ascospores by their poor growth when germinated on minimal-agar medium. Nearly 1200 ascospores from the cross 26–7 × *albino a* were germinated on minimal agar and examined, but none was seen that looked like a typical enzyme-negative type. Thirty-nine sporelings which showed weak growth were transferred for further testing. Of these, all except one proved to be inviable (quite unlike typical *am* mutants), and the other was a wild type. These data, combined with earlier results on the same lines obtained by Pateman (1957), make it reasonable to regard the original mutant 1038 as having acquired a new allele am^{3a} in place of the original mutant allele am^3, 'allele' being used to mean a distinct inheritable state of a locus, and 'locus' to mean a small discrete chromosomal segment differentiated from neighbouring loci by function (Fincham, 1959*a*).

DISCUSSION

The present study brings the number of alleles at the *am* locus, known to determine distinct varieties of glutamic dehydrogenase, to three. This is in addition to 11 alleles associated with absence of any glutamic-dehydrogenase activity (Fincham, 1959*a*). The allele am^{21}, as reported by Fincham

(1957), determines the production of a form of the enzyme which tends to be inactive at 20°, but which can be reversibly activated, perhaps through a refolding of the enzyme molecule with unmasking of the active centre, by raising the temperature. The active form of the 2l enzyme seems to be more or less similar to the wild-type enzyme in its Michaelis constants, so far as information is available. The glutamic dehydrogenase determined by am^{3a} bears some resemblance to the 2l enzyme in being activated by mild heat; however, the activation is much less readily reversible, and can be partially accomplished through incubation with α-oxoglutarate in the absence of TPNH, a procedure which does not activate the 2l enzyme. When fully activated, the 3a enzyme still differs sharply from the wild-type and 2l varieties in showing abnormally high Michaelis constants for all substrates. It may be significant that the increases in Michaelis constants, as compared with the wild-type values, are much greater for glutamate, oxidized TPN and NH_4^+ ion, substrates which all carry a positive charge, than for TPNH and α-oxoglutarate, which do not. This may suggest that the peculiarity of the 3a enzyme may consist in an alteration in the charge on the enzyme molecule in the vicinity of the active centre. It should be remembered, however, that there is no evidence which would exclude the possibility that unionized ammonia, rather than NH_4^+ ion, is the immediate substrate of the enzyme.

If it is assumed that the amount of glutamic dehydrogenase, expressed as a proportion of the total cell protein, is the same in am^{3a} strains as it is in the wild type, we must suppose that the 3a enzyme has a considerably higher turnover number for the back reaction (glutamate synthesis) than has the wild-type enzyme, even though much higher substrate concentrations are required to realize the maximum reaction velocity. It thus seems possible that, under conditions of high nutrition, am^{3a} might rank as a progressive mutant allele, in the sense of having a selective advantage over the original wild-type gene am^+. Rather surprisingly, it has not so far been possible to demonstrate any deleterious effect of am^{3a} on growth even under conditions of limited nitrogen supply.

It seems very unlikely that glutamic dehydrogenases from different sources act through basically different mechanisms. However, the compulsory substrate binding order of TPNH, NH_4^+ ion, α-oxoglutarate, which seems to follow from Frieden's (1959*c*) data for the ox-liver enzyme, does not seem very consistent with some of the substrate-activation effects which have been noted in the various *Neurospora*-enzyme types. Thus the 2l enzyme is activated by incubation with TPNH and α-oxoglutarate (both being necessary) in the absence of more than a trace of ammonium salt (Fincham, 1957), and the present study shows that α-oxoglutarate in the absence of either TPNH or ammonium salt can have a marked activating effect on the 3a enzyme. It seems possible, but unlikely, that these activating effects of TPNH and α-oxoglutarate have nothing to do with their substrate function. The lack of any strong effect, with the 3a enzyme, on the apparent Michaelis constant for one substrate, of variations in the concentrations of the other substrates, is more consistent with a random substrate-binding order than with the type of compulsory binding order deduced by Frieden. It may well be that random substrate binding, on the one hand, and a compulsory binding order, on the other, represent limiting cases which are seldom perfectly realized in practice; the combination of an enzyme with one substrate may be weakly or strongly affected by its combination with other substrates without the binding order being completely determined.

In addition to the three known alleles (am^+, am^{2l} and am^{3a}) which produce distinct active varieties of glutamic dehydrogenase, it is probable that several of the other known alleles produce inactive forms of the enzyme molecule. That this may be so is suggested particularly by the fact that, in two cases, pairs of *am* alleles which are individually incapable of promoting the formation of any sort of active glutamic dehydrogenase, can form active, though abnormal, forms of the enzyme when present together in a heterocaryon (Fincham, 1959*b*). There is evidence, both from ultracentrifuge data (Frieden, 1959*a*, *b*) and from electron microscopy (Valentine, 1959), that ox-liver glutamic dehydrogenase consists of dissociable and re-associable subunits. If the same applies to the *Neurospora* enzyme, complementary interaction between individually enzyme-negative alleles can be pictured as occurring through association of different varieties of defective subunit which have defects which are functionally non-overlapping.

It is clearly very desirable, where genetically determined variations in the properties of an enzyme are found, that the structural bases of the variations should be determined. Information of this sort would not only lead to a fuller understanding of the problem of the genetic determination of protein structure, but might also, through a correlation of chemical changes in the enzyme molecule with changes in kinetic properties, illuminate the detailed mechanism of the enzyme action.

SUMMARY

1. A new *Neurospora crassa* mutant allele of the *am* locus, a chromosome segment already known to be concerned with glutamic-dehydrogenase forma-

tion, causes the formation of a distinct variety of this enzyme. The new allele, which was derived by mutation of the mutant allele am^3, itself associated with apparent complete absence of glutamic dehydrogenase, is called am^{3a}.

2. The glutamic dehydrogenase associated with am^{3a} (the 3a enzyme) shows abnormally high Michaelis constants for all substrates, particularly for glutamate, triphosphopyridine nucleotide and NH_4^+ ion.

3. The ratio of maximum velocity in the reverse reaction (glutamate synthesis) to that in the forward reaction is about ten times as great for the 3a enzyme as for the wild-type enzyme. Extracts of am^{3a} strains have much higher maximum velocities in the reverse reaction than have wild-type extracts.

4. The 3a enzyme is less stable to heat than wild-type glutamic dehydrogenase.

5. In many preparations the 3a enzyme differs from the wild-type enzyme in not showing full activity until it has been given a mild heat treatment (e.g. 38° for a few minutes). This activation can also be brought about, to some extent, by incubation of the enzyme in the presence of α-oxoglutarate. Heat-activated 3a enzyme does not revert readily to the inactive form when returned to room temperature.

6. The main differences between the 3a and wild-type enzymes are just as marked in 40-fold-purified preparations as in crude extracts, though it was occasionally found that purified 3a preparations did not require activation. The two kinds of enzyme retained their respective properties when mixed together.

7. A heterocaryon containing both am^+ (i.e. wild type at the *am* locus) and am^{3a} nuclei produced glutamic dehydrogenase, which behaved as if it were a mixture of the 3a and wild-type enzymes.

8. Two mutant alleles at the *am* locus, each associated with a quite distinct abnormal type of glutamic dehydrogenase, are now known. Tests are described which distinguish the two mutant enzymes from each other, and from the wild type.

REFERENCES

Alberty, R. A. (1953). *J. biol. Chem.* **75**, 1928.

Evans, H. J. & Nason, A. (1953). *Plant Physiol.* **28**, 233.

Fincham, J. R. S. (1957). *Biochem. J.* **65**, 721.

Fincham, J. R. S. (1959*a*). *Proc. 10th int. Congr. Genetics*, vol. 1, p. 355. University of Toronto Press.

Fincham, J. R. S. (1959*b*). *J. gen. Microbiol.* **21**, 600.

Fincham, J. R. S. (1960). *Advanc. Enzymol.* (in the Press).

Frieden, C. (1959*a*). *J. biol. Chem.* **234**, 809.

Frieden, C. (1959*b*). *J. biol. Chem.* **234**, 815.

Frieden, C. (1959*c*). *J. biol. Chem.* **234**, 2891.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Newmeyer, D. (1957). *J. gen. Microbiol.* **16**, 449.

Olsen, H. A. & Anfinsen, C. B. (1953). *J. biol. Chem.* **202**, 841.

Pateman, J. A. (1957). *J. Genet.* **55**, 444.

Pateman, J. A. & Fincham, J. R. S. (1958). *Heredity*, **12**, 317.

Strecker, H. J. (1955). In *Methods in Enzymology*, vol. 2, p. 220. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Valentine, R. C. (1959). *Nature, Lond.*, **184**, 1838.

Vogel, H. J. & Bonner, D. M. (1956). *Microb. Genet. Bull.* **13**, 42.

Biochem. J. (1960) **77**, 105

Pyrimidine Metabolism in Parasitic Flatworms

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β-Alanine and β-amino*iso*butyric acid have previously been reported as prominent free amino acids in several species of parasitic flatworms (Campbell, 1960*a*, *b*). These two β-amino acids were not detected in the free-living flatworms which were examined. Because of these findings it was thought that an investigation of the metabolic origin of these compounds might contribute to a better understanding of the biochemistry of parasitism.

When β-alanine and β-amino*iso*butyric acid were first reported (Campbell, 1960*a*) from cestodes, it was suggested that they might arise from uracil and thymine respectively via the reductive pathway of pyrimidine degradation which has been established *in vivo* by Fink and co-workers (Fink, Henderson & Fink, 1952; Fink, Cline, Henderson & Fink, 1956; Fink, McGaughey, Cline & Fink, 1956; Fink, 1956) and *in vitro* by Grisolia and co-workers (Grisolia & Wallach, 1955; Wallach & Grisolia, 1957; Grisolia & Cardoso, 1957; Caravaca & Grisolia, 1958) and others (Canellakis, 1956;

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Fritzson & Pihl, 1956; Campbell, 1958). Free pyrimidines, arising from the digestion of the nucleic acid (Wilson & Wilson, 1958) in the host's ingesta, are available to the intestinal parasites and possibly to all parasites in contact with host tissue, since pyrimidines may occur generally in tissues in the free form (Travaglini, Levenbook & Schultz, 1958).

In the present work it has been shown that uracil and thymine are taken up *in vitro* by the rat tapeworm, *Hymenolepis diminuta*, and metabolized to β-alanine and β-amino*iso*butyric acid respectively via the reductive pathway of pyrimidine degradation. Two factors which affect the uptake and presumably the metabolism of these two compounds in this parasite are glucose and starvation of the host. Evidence is presented which indicates that α-[1-^{14}C]alanine and a [^{14}C]organic acid, which is probably succinic acid, are end products of degradation of [2-^{14}C]uracil by *H. diminuta*. The formation of these compounds is interpreted as being due to carbon dioxide fixation.

EXPERIMENTAL

The rat tapeworm was maintained in the laboratory in male rats of the Wistar strain (Read, 1951). Adult worms were flushed from the host's small intestine, after it had been removed from the body, with Krebs–Ringer solution (Umbreit, Burris & Stauffer, 1957), pH 7·4, with 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) acid maleate (Gomori, 1948). The wet weight of the individual worms averaged 316 mg. The average dry weight was approx. 18% of this. The percentage of nitrogen was 1·52 ± 0·02, as determined on homogenates by a modification of the micro-Kjeldahl method (Lang, 1958).

Uptake of pyrimidines. The uptake of pyrimidines by individual parasites was studied by measuring the disappearance of these compounds from the medium during incubation. The worms were first incubated for 30 min.–1 hr. in Krebs–Ringer solution, pH 7·4, with 0·05 M-tris acid maleate buffer and then they were placed in the experimental flasks containing 10 ml. of the buffered solution plus the pyrimidine at 1 mM concentration. The flasks were stoppered with cotton wool plugs and the incubations were carried out at 37° with constant agitation. Samples of the medium were taken at appropriate times, diluted and the concentration of the pyrimidine was determined spectrophotometrically in the Beckman DU spectrophotometer. During the incubations the parasites elaborate a substance that absorbs ultraviolet light with an absorption spectrum which is essentially flat between 250 and 310 mμ. Partial compensation for this material was achieved by basing the calculations on the difference between $E_{\lambda\,max.}$ and either $E_{290\,m\mu}$ (for the pyrimidines) or $E_{304\,m\mu}$ (for orotic acid). Controls were also run in each experiment in which the pyrimidine was omitted from the incubation medium and the amount of absorption due to this material was determined and subtracted from the experimental values.

Cytosine, 5-hydroxymethylcytosine, 5-methylcytosine and orotic acid were obtained from the California Corporation for Biochemical Research. Uracil and thymine were commercial preparations and were recrystallized twice from water.

Chromatography. β-Alanine and β-amino*iso*butyric acid were extracted from the worms as described by Campbell (1960*a*, *b*). Two-dimensional chromatography of the free amino acid extracts was carried out on Whatman no. 52 paper by the method of Levy & Chung (1953), with the substitution of a butan-2-ol–formic acid–water (75:15:10, by vol.) system in the first dimension. The β-amino acids were estimated from these chromatograms by the method of Fowden (1951).

Uracil, dihydrouracil and carbamoyl-β-alanine were extracted from the parasites with 75% (v/v) ethanol. These compounds were separated by one- or two-dimensional ascending chromatography with Whatman no. 52 paper and the following solvent systems: butan-2-ol–formic acid–water, ethyl acetate–formic acid–water (60:5:35, by vol.; upper phase) and methanol–pyridine–water (80:4:20, by vol.). Uracil and dihydrouracil gave identical R_F values in all but the ethyl acetate system. These two compounds were separated in the other systems by eluting the uracil–dihydrouracil spot, hydrolysing it with 0·5 N-NaOH and rechromatographing the hydrolysate to separate the original dihydrouracil from the uracil as carbamoyl-β-alanine. Dihydrouracil and carbamoyl-β-alanine were detected on the chromatograms by acidic *p*-dimethylaminobenzaldehyde by the method of Fink (1956).

Dihydrouracil and dihydrothymine were obtained from the California Corporation for Biochemical Research. Carbamoyl-β-alanine and carbamoyl-β-amino*iso*butyric acid were synthesized from the corresponding dihydropyrimidine as described by Fink, McGaughey *et al.* (1956).

Ammonium salts of the organic acids were chromatographed on Whatman no. 1 paper. The solvent systems used were butan-2-ol–formic acid–water, methanol–pyridine–water, ethyl acetate–formic acid–water, propanol–conc. NH_3 sol. (70:30, v/v) and ethanol–conc. NH_3 soln.–water (80:5:15, by vol.). The indicator consisted of a mixture of methyl red and bromothymol blue in 7% formaldehyde (Block, Durrum & Zweig, 1958).

Isotopic techniques. [2-^{14}C]Uracil was obtained from Schwarz Laboratories Inc., New York, U.S.A. Unlabelled uracil was added to this as a carrier to give the final concentrations used. For studies on the metabolism of uracil, the parasites were incubated in the [2-^{14}C]uracil plus carrier as described above. The radioactive compounds formed from [2-^{14}C]uracil were extracted and separated by chromatography as described above. They were detected on the chromatograms by No-Screen X-ray film (Eastman Kodak Co.) or by the use of a gas-flow chromatogram scanner. The amount of radioactivity in each compound was determined by eluting it from a chromatogram, plating the eluate on an aluminium planchet as a thin sample and counting the sample in a gas-flow counter. The $^{14}CO_2$ formed in the reactions was trapped in 10% (w/v) NaOH. It was then precipitated as $BaCO_3$ in the presence of NH_4Cl. An ethanolic slurry of the $BaCO_3$ was then plated as an infinitely thick sample and counted with a Geiger–Müller tube.

RESULTS

Uptake of pyrimidines by Hymenolepis diminuta *in vitro.* Of the six most commonly occurring pyrimidines, only uracil, thymine and cytosine were

taken up in significant quantities by the parasites. Uptake of orotic acid, 5-methyl- or 5-hydroxymethyl-cytosine was not detected (Fig. 1). The physiological state of the host influenced the rate at which uracil and thymine were taken up. When the hosts were starved 24 hr. before collection of the parasites, the uptake of uracil *in vitro* was approximately one-half of that of the parasites taken from hosts fed without restriction (Fig. 2). Since cestodes have a specific carbohydrate requirement and are sensitive to the host's dietary intake (Read, 1959), an attempt was made to reverse the effect of host starvation by the addition of glucose to the incubation medium. This resulted in an approximately twofold increase in uracil uptake by parasites taken from either starved or unstarved hosts (Fig. 2). Identical results were obtained with thymine. The presence of glucose in the incubation medium did not increase the uptake of cytosine whereas host starvation did. This is the reverse of the effects obtained with uracil and thymine and would suggest that a different mechanism may be involved in the metabolism of this pyrimidine.

Formation of β-alanine and β-aminoisobutyric acid from uracil and thymine respectively by Hymenolepis diminuta. Parasites freshly removed from the rat host contain about 0·6 μmole each of β-alanine and β-amino*iso*butyric acid/g. of tissue. These concentrations remain relatively constant during incubation for 3–4 hr. in Krebs–Ringer solution, indicating that there is neither a very rapid turnover of these β-amino acids nor a high rate of their formation from endogenous sources. However, the concentrations of β-amino acids could be increased approximately five- to twenty-fold by incubating the parasites for 3 hr. in solutions of uracil, thymine, or the intermediate dihydropyrimidines or carbamoyl-β-amino acids (Table 1). The carbamoyl-β-amino acids were only approximately 50 % as effective as precursors of β-alanine and β-amino*iso*butyric acid when compared with uracil and dihydrouracil or thymine and dihydrothymine. This effect has previously been noted with rat-liver slices and has been explained on the basis of the lower permeability of the carbamoyl compounds into the intact cells (Fink, McGaughey *et al.* 1956).

Identification of the intermediates of degradation of [2-^{14}C]*uracil.* The marked increase in the concentration of β-amino acids in *H. diminuta* after incubation in uracil and thymine, or of the intermediates of their degradation, strongly suggested that the reductive pathway of pyrimidine degradation was present. Evidence that dihydropyrimidines and carbamoyl-β-amino acids are the normal inter-

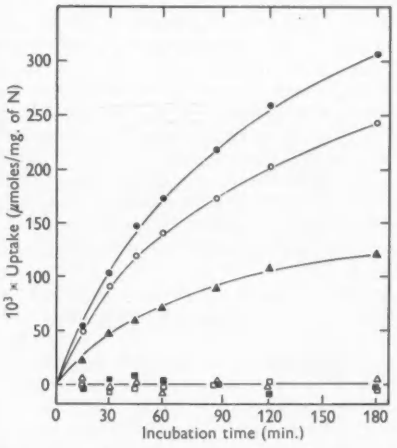

Fig. 1. Uptake of pyrimidines by *H. diminuta*. Each flask contained an individual parasite and 10 μmoles of the pyrimidine in Krebs–Ringer solution, pH 7·4, with 0·05 M-tris acid maleate buffer. Incubations were at 37° with constant agitation. The concentrations of the pyrimidines during the incubation were determined spectrophotometrically as described in the text. ●, Uracil; ○, thymine; ▲, cytosine; △, 5-methylcytosine; ■, 5-hydroxymethylcytosine; □, orotic acid.

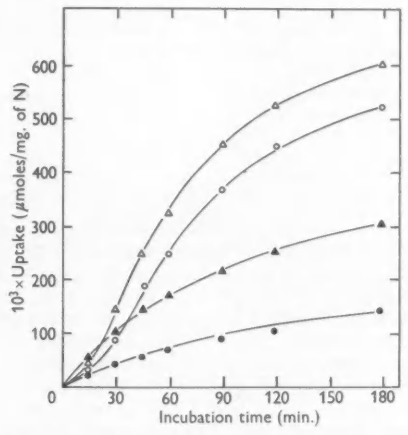

Fig. 2. Effect of host starvation on the uptake of uracil by *H. diminuta* and the reversal of this effect by glucose. Each flask contained an individual parasite and 10 μmoles of uracil in Krebs–Ringer solution, pH 7·4, with 0·05 M-tris acid maleate. Uracil was determined spectrophotometrically as described in the text. ▲, Parasites collected from rats fed without restriction (unstarved hosts); ●, parasites collected from rats which had been fasted 24 hr. before the time of collection (starved hosts); △, parasites from unstarved hosts with 1 mg. of glucose/ml. added to incubation solution; ○, parasites from starved hosts with 1 mg. of glucose/ml. added to incubation solution.

Table 1. *Formation of β-amino acids from pyrimidines and the intermediates of pyrimidine degradation by* Hymenolepis diminuta

Parasites were pooled to give 1 g. of tissue/flask. Each flask contained 150 μmoles of the substrate in Krebs–Ringer solution, pH 7·4, with tris acid maleate. Incubations were at 37° for 3 hr. The reaction was stopped with ethanol and the β-amino acids were extracted as described in the text. Results are given ±S.E.M. with the number of determinations in parentheses.

Treatment	Substrate	β-Alanine (μmoles/g. of tissue)	β-Amino*iso*butyric acid (μmoles/g. of tissue)
Freshly removed from host	—	0·60±0·04 (8)	0·65±0·08 (8)
Incubated for 3 hr. in Krebs–Ringer solution	—	0·85±0·13 (8)	0·63±0·08 (8)
Killed by heat (5 min. at 80°)	Each substrate tested*	0·95±0·40 (6)	0·68±0·25 (6)
Incubated for 3 hr.	Uracil	16·90±0·29 (4)	1·50±0·48 (4)
	Dihydrouracil	19·30±0·93 (4)	1·10±0·01 (4)
	Carbamoyl-β-alanine	6·80±0·60 (4)	0·60±0·01 (4)
	Thymine	0·90±0·01 (4)	11·10±0·50 (4)
	Dihydrothymine	1·50±0·01 (4)	10·50±0·63 (4)
	Carbamoyl-β-amino*iso*butyric acid	0·80±0·01 (4)	4·30±0·35 (4)

* These controls consisted of heat-killed parasites plus each substrate tested. Since there was little variation, the data were pooled from all the experiments for statistical analyses.

Table 2. *R_F values on Whatman no. 52 paper of known dihydrouracil and carbamoyl-β-alanine and two radioactive compounds formed from* [2-^{14}C]*uracil by* Hymenolepis diminuta

Solvent	R_F	
	Dihydrouracil	Carbamoyl-β-alanine
Butan-2-ol–formic acid–water (75:15:10, by vol.)	0·63	0·76
Ethyl acetate–formic acid–water (60:5:35, by vol.; upper phase)	0·45	0·52
Methanol–pyridine–water (80:4:20, by vol.)	0·54	0·63

mediates between the pyrimidines and the β-amino acids involved the chromatographic isolation and identification of radioactive dihydrouracil and carbamoyl-β-alanine after incubation of the parasites in [2-^{14}C]uracil. This was carried out in a typical experiment as follows. The parasites were pooled to give approximately 1 g. of tissue/flask. They were then incubated for 3 hr. at 37° in Krebs–Ringer solution, pH 7·4, containing 30 μmoles of [2-^{14}C]-uracil (specific activity 31 828 counts/min./μmole). At the end of the incubation they were quickly removed from the flask, washed and homogenized in 3 ml. of 75% (v/v) ethanol. The precipitate was removed by centrifuging and 200 μl. of this supernatant was spotted on to Whatman no. 52 paper for chromatography. Since the concentrations of dihydrouracil and carbamoyl-β-alanine were low in these extracts, only faint colours were given with acidic *p*-dimethylaminobenzaldehyde. To identify these compounds, 200 μl. of the extract to which had been added 1–2 μmoles each of known dihydrouracil and carbamoyl-β-alanine was chromatographed alongside the untreated extracts. The chromatogram was then exposed to X-ray film for several days. After the radioautograph had been developed, the chromatogram was sprayed with *p*-dimethylaminobenzaldehyde to detect the carbamoyl-β-alanine. It was then treated with 0·5N-NaOH and resprayed with *p*-dimethylaminobenzaldehyde to locate the dihydrouracil. This procedure was repeated with three different solvent systems and the R_F values of known dihydrouracil and carbamoyl-β-alanine and the radioactive compounds in the extracts, with identical properties with respect to their reaction with *p*-dimethylaminobenzaldehyde, are given in Table 2. The colour reaction of these compounds on the chromatograms and the radioactivity were found to coincide perfectly; tracings of typical radioautographs of the extracts and of the incubation medium are presented in Fig. 3.

Identification of the end products of degradation of [2-^{14}C]*uracil.* The formation of $^{14}CO_2$ from [2-^{14}C]-uracil has previously been used as a criterion for the reductive degradation of uracil (Canellakis, 1956). As shown in Fig. 4, this also takes place in *H. diminuta*.

In addition to carbon dioxide, dihydrouracil and carbamoyl-β-alanine, there are two other major radioactive compounds formed from [2-^{14}C]uracil, which, as described below, appear to be α-alanine and succinic acid. As shown in Table 3 and Fig. 5, these two compounds together account for approximately 60% of the recovered radioactivity in the extracts after incubation for 3 hr. The compound designated as α-alanine was separated from the β-alanine by streaking 300–500 μl. portions of the

extracts on Whatman no. 52 paper and developing the paper with butan-2-ol–formic acid–water. Radioautographs were then made and the radioactive area was eluted. The eluate was concentrated and portions were chromatographed with and without known α-alanine being added to them. Known α-alanine was also run alongside these extracts. Radioautographs were then made of these chromatograms and the radioactivity and ninhydrin reaction were found to coincide. The R_F values obtained for known alanine and the alanine isolated from the worm extracts are given in Table 4.

Table 3. *Distribution of the radioactivity from* [2-^{14}C]*uracil between the compounds, isolated from extracts of* Hymenolepis diminuta

After incubation for 3 hr. in [2-^{14}C]uracil the parasites were extracted and the radioactive compounds formed were isolated by chromatography as described in the text. The percentage of the radioactivity present in each compound was determined by eluting the compound from a one-dimensional chromatogram and counting a sample of the eluate with a gas-flow counter.

Compound	Radioactivity (%) Butan-2-ol–formic acid–water	Ethyl acetate–formic acid–water	Methanol–pyridine–water
Alanine	15·1	15·9	15·2
'Succinic acid'	43·9	43·4	44·1
Carbamoyl-β-alanine	4·1	3·9	4·1
Dihydrouracil	5·1	4·2	4·5
Uracil	31·3	32·0	31·3
Unaccounted for	0·5	0·6	0·8

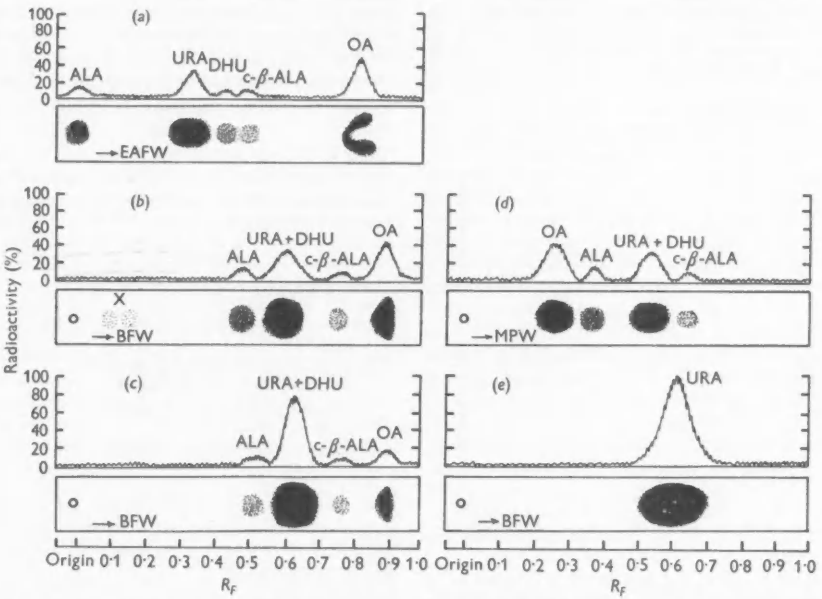

Fig. 3. Tracings of radioautographs made from chromatograms of the 75% ethanol extracts of parasites incubated in [2-^{14}C]uracil and of the incubation medium. Above each chromatogram is a tracing of the record obtained from a gas-flow chromatogram scanner which indicates the approximate percentage of the total recovered radioactivity in each compound. The extraction and chromatography is as described in the text. (*a*), (*b*) and (*d*) represent extracts chromatographed in three solvent systems: ethyl acetate–formic acid–water (EAFW), butan-2-ol–formic acid–water (BFW) and methanol–pyridine–water (MPW). (*c*) represents a sample of the incubation medium and (*e*) an extract of parasites which had been killed by heating (5 min. at 80°). ALA, α-Alanine; URA, uracil; DHU, dihydrouracil; c-β-ALA, carbamoyl-β-alanine; OA, organic acid; X, unidentified compounds which were not consistently detected.

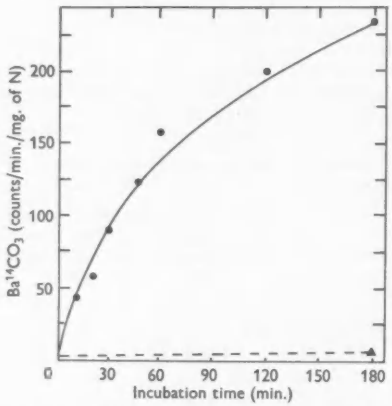

Fig. 4. Formation of $^{14}CO_2$ from [2-^{14}C]uracil by *H. diminuta*. The reaction was carried out in Warburg vessels containing 10% NaOH in the centre well. At time 0, 0·75 ml. of 0·02 M-[2-^{14}C]uracil was added to the incubation medium (total volume 3·25 ml.) and the incubation was carried out at 37°. The reaction was stopped at the indicated time intervals with 10% (w/v) trichloroacetic acid. The trapped CO_2 was precipitated as $BaCO_3$ and counted with a Geiger–Müller tube. ●, Experimental; ▲, killed by heat (5 min. at 80°).

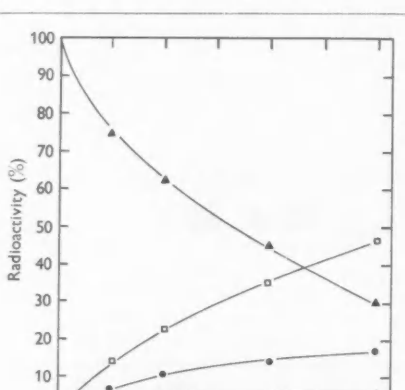

Fig. 5. Percentage of the radioactivity in extracts of *H. diminuta* present in uracil, dihydrouracil, carbamoyl-β-alanine, alanine and 'succinic acid' during incubation for 3 hr. in [2-^{14}C]uracil. The conditions of the incubation and extraction of the compounds are as described in the text. The percentage of the radioactivity in each compound was determined by eluting the compound from a one-dimensional chromatogram (developed with ethyl acetate–formic acid–water) and counting a sample of the eluate with a gas-flow counter. ▲, Uracil; □, 'succinic acid'; ●, α-alanine; △, dihydrouracil; ○, carbamoyl-β-alanine.

The radioactivity of the alanine was localized in the carboxyl group in the following manner. An eluate containing 2200 counts/min. of the alanine in 2 ml. was caused to react with 1 ml. of Fowden's (1951) ninhydrin reagent at pH 5·0, in a closed vessel at 100° for 30 min. The CO_2 formed was trapped in 10% NaOH and 2036 counts/min. were recovered as $Ba^{14}CO_3$.

Preliminary experiments indicated that the compound which accounts for over 40% of the recovered radioactivity in the extracts after incubation for 3 hr. in [2-^{14}C]uracil was an organic acid (designated as OA in Fig. 3). This compound is the first compound in which activity could be detected. It was easily detectable by radioautography after incubation for 5 min. This compound was chromatographically isolated as described above for the alanine. Chromatographic comparisons with several known acids suggested that it might be succinic acid. Portions of the eluate were neutralized with NH_3 solution and were chromatographed with and without added succinic acid neutralized in the same manner. When radioautographs of these were prepared, the radioactivity and indicator reaction were found to coincide exactly. The R_F values of the radioactive organic acid isolated from the parasite extracts and known succinic acid are given in Table 5.

Table 4. *R_F values on Whatman no. 52 paper of known α-alanine and radioactive α-alanine isolated from extracts of* Hymenolepis diminuta *after incubation in* [2-^{14}C]*uracil*

Solvent	R_F
m-Cresol–phenol–borate buffer, pH 9·3 (60:30:15, by vol.)	0·36
Butan-2-ol–formic acid–water (75:15:10, by vol.)	0·45
Ethyl acetate–formic acid–water (60:5:35, by vol.; upper phase)	0·02
Methanol–pyridine–water (80:4:20, by vol.)	0·61
Propanol–conc. NH_3 soln. (70:30, v/v)	0·51

Table 5. *R_F values on Whatman no. 1 paper of known succinic acid and the radioactive organic acid isolated from* Hymenolepis diminuta *after incubation in* [2-^{14}C]*uracil*

Solvent	R_F
Propanol–conc. NH_3 soln. (70:30, v/v)	0·14
Ethanol–conc. NH_3 soln.–water (80:5:15, by vol.)	0·18
Butan-2-ol–formic acid–water (75:15:10, by vol.)	0·75
Ethyl acetate–formic acid–water (60:5:35, by vol.; upper phase)	0·81
Methanol–pyridine–water (80:5:20, by vol.)	0·25

Traces of other radioactive compounds were occasionally detected on radioautographs of the parasite extracts (Fig. 3, X) but were not identified. [^{14}C]Urea is one of the major products from [2-^{14}C]thymine in the rat (Fink, McGaughey *et al.* 1956). To determine if any [^{14}C]urea was formed by these parasites from [2-^{14}C]uracil, all the radioactive spots on the chromatograms were eluted and treated with urease. No $^{14}CO_2$ production was detected with any of the spots whereas [^{14}C]urea added to the worm extracts and carried through the same procedure of chromatography, elution and urease treatment resulted in the formation of $^{14}CO_2$.

DISCUSSION

The evidence presented indicates that reductive degradation of uracil and thymine via either dihydrouracil and carbamoyl-β-alanine or dihydrothymine and carbamoyl-β-amino*iso*butyric acid to form β-alanine or β-amino*iso*butyric acid respectively is an important pathway in the rat parasite *H. diminuta*. That this pathway may be present in several other parasitic flatworms is indicated by the rather wide occurrence of β-alanine and β-amino*iso*butyric acid in these forms (Campbell, 1960*a*, *b*).

Previous work with the reductive pathway of thymine degradation *in vivo* by Fink and co-workers (Fink *et al.* 1952; Fink, McGaughey *et al.* 1956; Fink, Cline *et al.* 1956) has shown that, in rat-liver slices, various radioactive compounds are formed, in addition to dihydrothymine and carbamoyl-β-amino*iso*butyric acid, depending upon the position of the radioactive carbon in the thymine molecule. The radioactive compounds identified from methyl-labelled thymine were 5-hydroxymethyluracil, alanine and glucose. With [2-^{14}C]thymine these investigators have reported radioactive 5-hydroxymethyluracil, urea, uracil-5-carboxylic acid and carbon dioxide. In *H. diminuta*, $^{14}CO_2$ α-[1-^{14}C]-alanine and a [^{14}C]organic acid, which is probably succinic acid and will be referred to as such subsequently, are the major end products identified from metabolism of [2-^{14}C]uracil. The most tenable explanation for the formation of the last-named two compounds involves a mechanism of carbon dioxide fixation. Carbon dioxide fixation by animal parasites has previously been reported by Fairbairn (1954) in the roundworm of chickens, *Heterakis gallinae*, and more recently by Saz & Vidrine (1959) in muscle of the roundworm of swine, *Ascaris lumbricoides*. *Heterakis* was found to fix carbon dioxide into propionic acid and a non-volatile acid which is probably succinic acid. In *Ascaris* muscle, the mechanism involves an initial fixation into pyruvic acid followed by a reduction to form succinic acid. It was also found that in *Ascaris* muscle succinic acid may be decarboxylated to form propionic acid. Hammen & Wilbur (1959) have shown that in the oyster there is an initial fixation of carbon dioxide into propionic acid to form succinic acid. The mechanism of the formation of the [^{14}C]-succinic acid and α-[1-^{14}C]alanine in *H. diminuta* from [2-^{14}C]uracil is at present not known. The fixation of $^{14}CO_2$ from the uracil into the carboxyl group of the succinic acid followed by oxidation and decarboxylation to form pyruvic acid is one pathway which could account for the labelling of the carboxyl group of alanine, since the pyruvic acid $\rightleftharpoons$ alanine transaminase system is present in this parasite (Aldrich, Chandler & Daugherty, 1954).

The increased uptake of uracil or thymine in the presence of glucose might be due to an increased metabolism of these compounds. Glucose did not increase the uptake of cytosine, which is apparently not degraded by animal tissues (Fink, McGaughey *et al.* 1956). Read (1956) has shown that glucose increases both the oxygen consumption and lactic acid production by these parasites. Host starvation results in a greatly diminished polysaccharide content and a decreased rate of acid production. The amount of acid produced is as follows: parasites from starved hosts plus glucose > from unstarved hosts plus glucose > from unstarved hosts > from starved hosts. Host starvation was also found to increase the amount of glycogen synthesis by the parasites in the presence of glucose (Read, 1956; Read & Rothman, 1957). Although parasites from starved hosts produce more lactic acid in the presence of glucose, they have a lower rate of glucose uptake (Phifer, 1960). The uptake of uracil then corresponds to the rate of glucose uptake. The time lag before glucose has its effect (Fig. 2) indicates that the increased rate of uracil uptake may be dependent upon glucose metabolism. Preliminary experiments show that uracil is not transported against a concentration gradient by *H. diminuta*. Similar results have been obtained with rat and hamster intestinal preparations (Wilson & Wilson, 1958). This indicates that the effect of glucose and host starvation reflects an effect on the metabolism of this compound and not on some transport mechanism. This effect may be one of an increased or decreased supply of reducing compounds or adenosine triphosphate.

SUMMARY

1. The rat tapeworm, *Hymenolepis diminuta*, has been found to take up uracil, thymine and cytosine *in vitro*. The addition of glucose to the medium results in a marked increase in the uptake of uracil and thymine, but not of cytosine.

2. Uracil and thymine are degraded to β-alanine and β-amino*iso*butyric acid respectively by this parasite. The degradation of [2-^{14}C]uracil results in

the formation of radioactive dihydrouracil, carbamoyl-β-alanine, carbon dioxide and, in addition, α-[1-^{14}C]alanine, some unidentified compounds and an organic acid which is chromatographically identical with succinic acid.

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REFERENCES

Aldrich, D. V., Chandler, A. C. & Daugherty, J. W. (1954). *Exp. Parasit.* **3**, 173.
Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 218. New York: Academic Press Inc.
Campbell, J. W. (1960*a*). *Exp. Parasit.* **9**, 1.
Campbell, J. W. (1960*b*). *Biol. Bull., Wood's Hole* (in the Press).
Campbell, L. L., jun. (1958). *J. biol. Chem.* **233**, 1236.
Canellakis, E. S. (1956). *J. biol. Chem.* **221**, 315.
Caravaca, J. & Grisolia, S. (1958). *J. biol. Chem.* **231**, 357.
Fairbairn, D. (1954). *Exp. Parasit.* **3**, 52.
Fink, K. (1956). *J. biol. Chem.* **218**, 9.
Fink, K., Cline, R. E., Henderson, R. B. & Fink, R. M. (1956). *J. biol. Chem.* **221**, 425.
Fink, K., Henderson, R. B. & Fink, R. M. (1952). *J. biol. Chem.* **197**, 441.
Fink, R. M., McGaughey, C., Cline, R. E. & Fink, K. (1956). *J. biol. Chem.* **218**, 1.
Fowden, L. (1951). *Biochem. J.* **48**, 327.
Fritzson, P. & Pihl, A. (1956). *J. biol. Chem.* **226**, 229.
Gomori, G. (1948). *Proc. Soc. exp. Biol., N.Y.*, **68**, 354.
Grisolia, S. & Cardoso, S. S. (1957). *Biochim. biophys. Acta*, **25**, 430.
Grisolia, S. & Wallach, D. P. (1955). *Biochim. biophys. Acta*, **18**, 449.
Hammen, C. S. & Wilbur, K. M. (1959). *J. biol. Chem.* **234**, 1268.
Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
Phifer, K. O. (1960). *J. Parasit.* **46**, 145.
Read, C. P. (1951). *Exp. Parasit.* **1**, 1.
Read, C. P. (1956). *Exp. Parasit.* **5**, 325.
Read, C. P. (1959). *Exp. Parasit.* **8**, 365.
Read, C. P. & Rothman, A. H. (1957). *Exp. Parasit.* **6**, 280.
Saz, H. J. & Vidrine, A., jun. (1959). *J. biol. Chem.* **234**, 2001.
Travaglini, E. C., Levenbook, L. & Schultz, J. (1958). *Exp. Cell Res.* **15**, 62.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, 3rd ed., p. 149. Minneapolis, Minn.: Burgess Publishing Co.
Wallach, D. P. & Grisolia, S. (1957). *J. biol. Chem.* **226**, 277.
Wilson, T. H. & Wilson, D. W. (1958). *J. biol. Chem.* **233**, 1544.

Biochem. J. (1960) **77**, 112

Immunological Properties of Exopenicillinase Synthesized by *Bacillus cereus* 569/H in the Presence of Amino Acid Analogues

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Previous experiments with *Staphylococcus aureus* 524 have shown that the replacement in the growth medium of the essential amino acid, arginine, by the structural analogue canavanine led to continued protein synthesis, but no production of active forms of phosphatase, β-galactosidase, hyaluronidase nor of a lytic enzyme which digested cell-wall preparations from *Micrococcus lysodeikticus*. To explain this finding it was suggested that protein synthesized in the presence of canavanine contained that amino acid in place of arginine and that this resulted in inactive forms of the enzymes (Richmond, 1959*a*). This possibility was supported by the detection of canavanine in the protein fraction of organisms incubated under these conditions (Richmond, 1959*b*).

It would seem that the one feasible method of testing this hypothesis would be by detecting an immunological cross-reaction between the inactive enzyme protein and a specific antibody against the normal enzyme. Unfortunately specific antisera against the enzymes studied in *S. aureus* were not available nor did it seem practicable to purify these enzymes to the degree necessary for the preparation of such sera. Accordingly, the effect of canavanine was studied on penicillinase formation by *Bacillus cereus* 569 (inducible strain) and *B. cereus* 569/H (constitutive strain). This system was

chosen as the relevant enzyme has been crystallized (Kogut, Pollock & Tridgell, 1956), its biosynthesis has been investigated extensively (Pollock, 1956*b*; Pollock & Kramer, 1958) and highly specific antisera were available together with techniques for their use in the isolation of small quantities of pure exopenicillinase (Pollock, 1956*a*; Pollock & Kramer, 1958). Further, the rate of protein 'turnover' has been measured in the constitutive strain (Urbá, 1959).

In addition to canavanine, the phenylalanine analogue *p*-fluorophenylalanine has also been used in this work, since it has been studied in detail (Munier & Cohen, 1956, 1959; Cohen & Munier, 1959; Cowie, Cohen, Bolton & de Robichon-Szulmajster, 1959) and shown to replace phenylalanine but not tyrosine in the protein fraction of *Escherichia coli*. Further, a supply of *p*-fluorophenyl[β-^{14}C]alanine was available, whereas no radioactive isomers of canavanine could be readily obtained.

This paper describes preliminary experiments on the effect of canavanine and *p*-fluorophenylalanine on growth and enzyme formation by *B. cereus* 569 and 569/H, together with a detailed description of the immunological properties of the penicillinase formed in the presence of these unnatural amino acids. Unlike *S. aureus* 524, this strain of *B. cereus* is not exacting for either arginine or phenylalanine and, as a result, the effect of the externally added amino acid analogues may be altered by the presence of the endogenously synthesized normal amino acids. To assess the importance of these possibilities an analysis of the effect of *p*-fluorophenylalanine on cell metabolism and penicillinase synthesis in *B. cereus* 569/H was carried out and is the subject of the subsequent paper (Richmond, 1960). Attempts to interpret the immunological differences exhibited by the penicillinase formed in the presence of *p*-fluorophenylalanine in the light of the effect of this amino acid on growth and penicillinase synthesis will be deferred to the following paper.

MATERIALS AND METHODS

Organisms. The inducible strain (569) and the constitutive mutant strain (569/H) of *B. cereus* used in these studies have been described previously (Kogut *et al.* 1956; Pollock, 1956*a*, *b*). Induction of the inducible strain (569) was carried out where required with 1 unit of benzylpenicillin/ml. as described by Pollock (1952).

Media. The casein-hydrolysate medium has been described by Kogut *et al.* (1956). The fully defined amino acid medium (18AA medium) was the same as that used for growth of *S. aureus* 524SC (Richmond, 1959*a*), except that nicotinic acid and thiamine hydrochloride, being unnecessary for the growth of *B. cereus*, were omitted. For specific purposes 18AA medium was modified by omitting arginine [17AA(– Arg) medium] or phenylalanine [17AA(– Phe) medium].

Amino acids. I am indebted to Dr J. Mandelstam for a gift of L-canavanine, which had been prepared from jack beans. The β-(*p*-fluorophenyl)alanine and further samples of L-canavanine sulphate were obtained from L. Light and Co. Ltd, Colnbrook, Bucks, England. DL-β-(*p*-Fluorophenyl)[β-^{14}C]alanine was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, (Seine et Oise), France. It was diluted before use with unlabelled DL-β-(*p*-fluorophenyl)alanine to a specific activity of 0·17 μc/μmole.

Anti-exopenicillinase. Two preparations of immune-rabbit γ-globulin with similar neutralization characteristics were provided by Dr M. R. Pollock for use in these studies. The first (prepared from rabbit 362; see Pollock, 1956*a*) was used in the majority of experiments and had a titre of about 10^4 neutralization units/ml. The second preparation was made by Pollock & Kramer (1958) and had a titre of $1{\cdot}2 \times 10^5$ neutralization units/ml. Both preparations were diluted in 0·9% sodium chloride solution containing 0·8 mM-oxine to a titre of about 450 neutralization units/ml. and stored at −20°.

Preparation of bacteria for experimental purposes. About 0·2 ml. of a spore suspension (approx. 3×10^9 spores/ml.) was used to inoculate 20 ml. of casein-hydrolysate medium and incubated in stationary culture at 35° overnight. After 16 hr. the culture was diluted tenfold with fresh casein-hydrolysate medium and shaken at the maximum rate (86 cyc./min.; 1 ft. throw) of a graded shaker (Kantorowicz, 1951) until growth was exponential. When the culture had reached a density of 0·2–0·4 mg. dry wt. of bacteria/ml., the organisms were removed by centrifuging, washed in 0·9% sodium chloride solution and resuspended in the experimental media. If the subsequent experiments were to be carried out in amino acid media, organisms grown up in casein-hydrolysate medium were given a period of growth in 18AA, 17AA(– Arg) or 17AA(– Phe) medium, as required, to accustom them to the change in growth conditions.

Crude exopenicillinase. Crude preparations of both normal and abnormal penicillinase consisted of the supernatant growth medium remaining after removal of the organisms from appropriate cultures by centrifuging. These preparations were diluted for enzyme estimation or neutralization experiments in 1% of gelatin in water containing 0·8 mM-oxine to give a final concentration of about 20–30 enzyme units/ml. For certain purposes crude enzyme preparations were freed of low-molecular-weight contaminants by dialysis at 2° against four changes of 50 vol. of mM-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0.

Preparation of cell fractions. The 'protein + cell wall' fraction of organisms was prepared as described for *S. aureus* 524 SC by Richmond (1959*a*, *b*). Cell-wall preparations were made by the method of Mandelstam & Rogers (1959). Free amino acids were liberated from these fractions by hydrolysis in 6N-HCl for 16 hr. at 105° in a sealed tube. The amino acids were separated by descending chromatography in butanol–acetic acid–water (60:10:27, by vol.). The locations of radioactive spots on the chromatograms were detected by radioautography as described by Roberts, Abelson, Cowie, Bolton & Britten (1955).

Assay systems. The technique of penicillinase assay and the titration of anti-exopenicillinase antiserum have been described elsewhere (Kogut *et al.* 1956; Pollock, 1956*a*).

RESULTS

Effect of canavanine and p-*fluorophenylalanine on growth and enzyme formation*

Cultures of *B. cereus* 569 and 569/H grew exponentially up to a density of about 0·8 mg. dry wt. of bacteria/ml. in the basal 18AA, 17AA(−Phe) and 17AA(−Arg) media used in these experiments. In 18AA and 17AA(−Phe) media the mean generation time of the organisms was about 50 min. whereas in 17AA(−Arg) medium it was 60–65 min.

Fig. 1 shows the growth curves obtained when organisms growing exponentially in 18AA medium were centrifuged, washed in 0·9 % sodium chloride solution and immediately resuspended in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. or in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. The culture incubated in the presence of canavanine grew only for about 60 min., but that in *p*-fluorophenylalanine increased at a lower exponential rate than the control for about 60 min., before becoming approximately linear with respect to time. The 'linear' growth phase may continue in the presence of *p*-fluorophenylalanine until the culture reaches a density of greater than 0·8 mg. dry wt. of organisms/ml.

Cultures of *B. cereus* 569/H growing exponentially in fully synthetic amino acid medium synthesized penicillinase at a constant differential rate and the total penicillinase activity of cultures under these conditions may be ascribed to three main penicillinase fractions. An exocellular fraction (α- or exo-

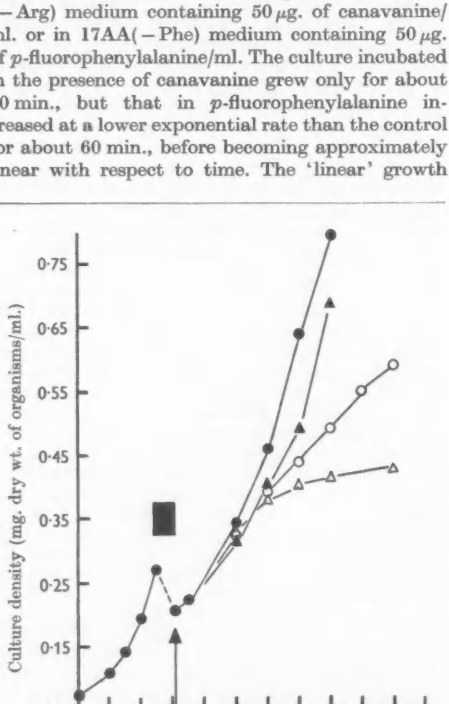
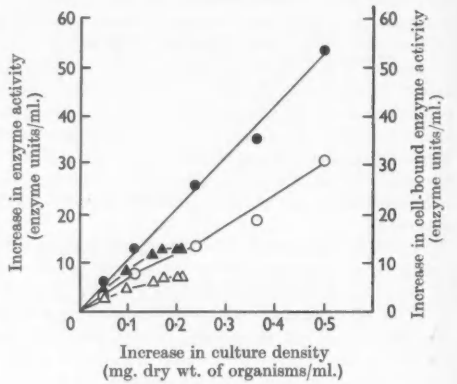


Fig. 1. Effect of canavanine or *p*-fluorophenylalanine on the growth of cultures of *Bacillus cereus* 569/H. ●, 18AA or 17AA(−Phe) medium; ▲, 17AA(−Arg) medium; ○, 17AA(−Phe + *p*-fluorophenylalanine) medium; △, 17AA(−Arg + canavanine) medium. *p*-Fluorophenylalanine or canavanine was added at arrow. Solid rectangle indicates time taken to centrifuge organisms and to resuspend them in the experimental media.



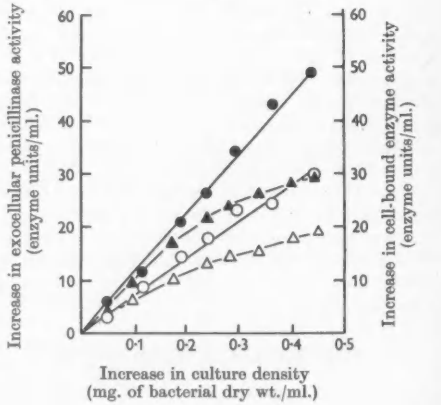


Fig. 2. Effect of (*a*) canavanine and (*b*) *p*-fluorophenylalanine on the differential rate of appearance of penicillinase fractions in cultures of *B. cereus* 569/H. (*a*) Exopenicillinase: ●, 17AA(−Arg) medium; ▲, 17AA(−Arg + canavanine) medium. Total cell-bound penicillinase: ○, 17AA(−Arg) medium; △, 17AA(−Arg + canavanine) medium. (*b*) Exopenicillinase: ●, 17AA(−Phe) medium; ▲, 17AA(−Phe + *p*-fluorophenylalanine) medium. Total cell-bound penicillinase: ○, 17AA(−Phe) medium; △, 17AA(−Phe + *p*-fluorophenylalanine) medium.

penicillinase) and a cell-bound fraction, about 40% of which is α-penicillinase that has been adsorbed on to the cell surface [the β-penicillinase of Pollock (1956*b*)] and the remaining 60% an immunologically and physicochemically distinct penicillinase (γ-penicillinase). The relative proportions of these fractions do not vary significantly during normal exponential growth (see Pollock, 1952, 1956*b*). As both canavanine and *p*-fluorophenylalanine have a marked effect on growth, they would be expected to have an effect on the absolute amount of penicillinase appearing in the culture medium. To determine whether either amino acid analogue inhibited penicillinase formation to a greater extent than it inhibited growth or whether there was any disproportionate effect on any of the penicillinase fractions, the differential rate of synthesis of total cell-bound and exocellular enzyme was followed in cultures incubated in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. and in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. The differential rate of formation of both penicillinase fractions is markedly impaired in the presence of canavanine (Fig. 2*a*). The effect may be observed within 15 min. of addition of the analogue and affects the two enzyme fractions to the same extent. On the other hand, the rate of synthesis in the presence of *p*-fluorophenylalanine (Fig. 2*b*) is only slightly affected within 45 min. of addition of the analogue and thereafter falls to about 40% of the control rate. This lower rate of penicillinase synthesis in the presence of *p*-fluorophenylalanine may continue for 2–3 hr. under these conditions. Further, if organisms incubated in this way for 90 min. are centrifuged and immediately resuspended in fresh 17AA(−Phe) medium containing *p*-fluorophenylalanine, the turbidity of the culture continues to increase linearly with time and exopenicillinase is then being synthesized at about 40% of the differential rate attained in the control.

Protection from canavanine and p-*fluorophenylalanine inhibition with arginine and phenylalanine*

The addition of 50 μg. of canavanine or *p*-fluorophenylalanine/ml. to cultures growing exponentially in 'S' peptone broth (Pollock & Perret, 1951) or 18AA medium had no effect on the growth rate or the differential rate of enzyme synthesis. The presence of arginine in cultures containing canavanine, or of phenylalanine in those containing *p*-fluorophenylalanine, protected the organisms from inhibition. Fig. 3*a* shows the effect of adding arginine (5 μg./ml.) and Fig. 3*b* that of adding phenylalanine (5 μg./ml.) to cultures incubated for varying periods of time in 17AA(−Arg) medium

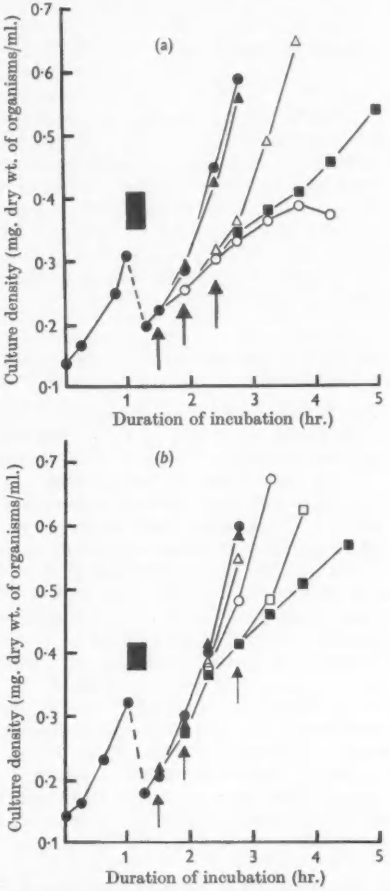


Fig. 3. Reversal of inhibitory effects on growth of (*a*) canavanine by arginine and (*b*) *p*-fluorophenylalanine by phenylalanine. (*a*) Organisms were grown in 17AA(−Arg) medium (●) for 1 hr. and then centrifuged and resuspended in experimental media (for duration of centrifuging see solid rectangle). Experimental media: ●, 17AA(−Arg) medium; ○, 17AA(−Arg+canavanine) medium (canavanine added at 1 hr. 20 min.); 17AA(−Arg+canavanine) medium with reversing concentrations of arginine added at intervals: ▲, at 1 hr. 30 min.; △, at 1 hr. 55 min.; ■, at 2 hr. 25 min. (see arrows). (*b*) Organisms were grown in 17AA(−Phe) medium for 1 hr. (●) and then centrifuged and resuspended in the experimental media (for duration of centrifuging see solid rectangle). Experimental media: ●, 17AA(−Phe) medium; ■, 17AA(−Phe+*p*-fluorophenylalanine) medium (*p*-fluorophenylalanine added at 1hr.30min.);17AA(−Phe+*p*-fluorophenylalanine) medium with reversing concentrations of phenylalanine added at intervals: ▲, at 1 hr. 30 min.; ○, at 1 hr. 55 min.; □, at 2 hr. 45 min. (see arrows).

containing 50 μg. of canavanine/ml. and 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. respectively. Arginine added at the same time as canavanine completely protects the culture from inhibition; delay in addition of arginine leads to an increased lag period before the resumption of exponential growth. On the other hand, addition of phenylalanine leads to resumption of the full exponential growth rate within 40 min. even when incubation in the presence of *p*-fluorophenylalanine has continued for 90 min. In all cases, restitution of exponential growth was accompanied by a normal differential rate of synthesis of all the penicillinase fractions.

Incorporation of canavanine and p*-fluorophenylalanine into the protein fraction of organisms*

In view of the reports of incorporation of canavanine into protein of *S. aureus* (Richmond, 1959*b*) and *p*-fluorophenylalanine into *E. coli* (Munier & Cohen, 1956, 1959; Cohen & Munier, 1959), and the inhibitory action of these two analogues on growth and enzyme formation reported above, it was of interest to see whether these compounds were incorporated into the protein fraction of *B. cereus*. Cultures grown for 90 min. in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. were precipitated with an equal volume of 10% trichloroacetic acid, containing 10 mg. of L-arginine hydrochloride/ml., and then fractionated as described by Richmond (1959*a*, *b*). In this way it was possible to show the presence of canavanine in the protein + cell-wall fraction of *B. cereus*. As canavanine cannot be detected in cell-wall preparations isolated from this strain after growth in canavanine, it seems likely that the analogue is incorporated in the protein fraction of the organisms.

The incorporation of *p*-fluorophenylalanine into the protein fraction of *B. cereus* was followed by growing cultures in 17AA(−Phe) medium containing *p*-fluorophenyl[β-^{14}C]alanine (final concentration 50 μg./ml. at 0·17 μc/μmole). The rate of incorporation of radioactivity increased during the first 45 min., thereafter reaching a constant rate with respect to time. The protein + cell-wall fraction was prepared from organisms grown under these conditions for 90 min., hydrolysed in 6N-HCl to liberate free amino acids and the presence of *p*-fluorophenylalanine was shown by radioautography after separating the hydrolysate by chromatography in butanol–acetic acid–water. The radioactive material obtained in this way was further identified as *p*-fluorophenylalanine by the 'finger-print' technique of Roberts *et al.* (1955). No other radioactive spot was detected in the hydrolysate prepared in this way.

Immunological properties of exopenicillinase synthesized in the presence of amino acid analogues

The results reported above imply therefore that the incorporation of the analogues into the protein fraction of the organisms interferes with the appearance of penicillinase activity to a greater extent than growth. An analysis of this problem is the subject of the succeeding paper; this paper is concerned with the immunological properties of the exopenicillinases formed in the presence of canavanine and *p*-fluorophenylalanine.

For convenience, the immunological character chosen for study was the behaviour of the enzyme in the neutralization reaction between enzyme and specific antiserum. This test corresponds to a constant antigen titration and its characteristics for normal exopenicillinase from *B. cereus* 569 and 569/H have been fully investigated by Pollock (1956*a*). As antiserum is added to normal enzyme (see Fig. 4), the enzymic activity of the enzyme–antibody mixture falls until about 5% of the original activity remains. The disappearance of activity is proportional to the amount of antiserum added except at low antibody–enzyme ratios where the neutralizing ability of the antiserum is slightly impaired. To obtain a quantitative description of this process, the following neutralization characteristics (see Fig. 4) have been measured.

(*a*) Plateau zone (A, Fig. 4). This is the range of antibody–enzyme ratios below which the antiserum does not show its maximum neutralizing titre. The width of the plateau zone is measured as the quantity of antiserum (in neutralization units) equivalent to the distance A in Fig. 4. To deter-

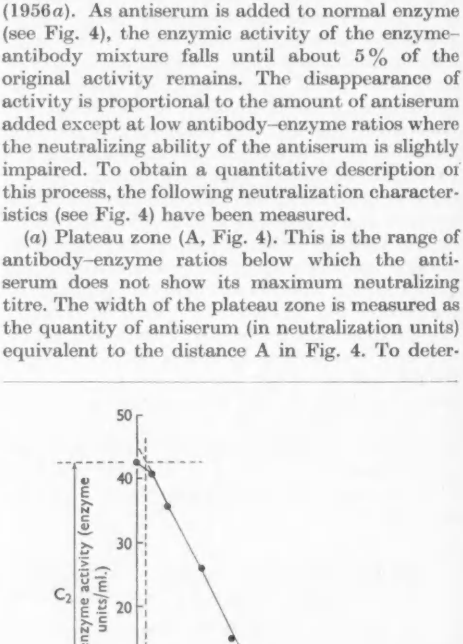


Fig. 4. Neutralization characteristics of normal exopenicillinase from *B. cereus* 569/H. For details see text.

mine this value exactly would require a large number of estimations and, consequently, the values quoted below are subject to large errors and are only approximate. In normal enzyme–antibody neutralization, with an initial enzyme titre of about 40 units/ml. the plateau zone corresponds to about 3·0 neutralization units.

(*b*) Neutralization phase (B_1, Fig. 4). This is the range of enzyme–antibody ratios lying between the plateau zone and the Equivalence Point (E, Fig. 4), over which the antiserum shows full neutralizing titre (measured in neutralization units/0·1 ml.). The maximum neutralization slope appears independent of initial enzyme concentration and is measured as C_2/B_1 (Fig. 4).

(*c*) Residual activity (C_1, Fig. 4). This is the amount of enzyme activity remaining at antibody–enzyme ratios greater than those required for maximum neutralization of enzyme. The percentage residual activity is 100 (C_1/C_2) (Fig. 4). Normal enzyme shows a residual activity of about 5–8 % of the initial enzyme titres.

Neutralization of enzyme synthesized in the presence of p-*fluorophenylalanine.* To test the neutralization characteristics of enzyme synthesized in the presence of *p*-fluorophenylalanine, organisms growing exponentially in 17AA(−Phe) medium were separated by centrifuging, washed and resuspended to a density of 0·2 mg. of bacteria/ml. in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. After incubation for 2 hr. (when the culture density is approx. 0·45 mg. dry wt. of bacteria/ml.), the organisms were removed by centrifuging and the supernatant liquid containing the exopenicillinase was mixed with one-third of its volume of 20 % (w/v) gelatin containing 1·6 mM-oxine for enzyme estimations. Normal exopenicillinase, prepared from a culture growing exponentially in 18AA medium, was diluted with fresh 18AA medium and oxine–gelatin to give approximately the same final titre as the material prepared in the presence of *p*-fluorophenylalanine. Fig. 5 compares the neutralization curves obtained when these enzyme preparations were titrated with anti-exopenicillinase serum. The enzyme synthesized in the presence of *p*-fluorophenylalanine differs from the normal in three respects. (1) The plateau zone is more marked with enzyme synthesized in the presence of *p*-fluorophenylalanine. In this experiment, the first 0·02 ml. of antiserum had little effect on the enzyme titre and the plateau zone, in this case, is equivalent to about 12–13 neutralization units/40 enzyme units, or about five times the normal. (2) The maximum neutralization slope of the antiserum (i.e. the slope of the neutralization curve during the neutralization phase) was about 55 % of that achieved with normal enzyme. (3) The residual activity after addition of 1·5 equivalents of antiserum (taking account of the lower neutralization titre) is about 1·2–1·4 times that found with normal enzyme.

The neutralization characteristics of varying concentrations of normal and analogue enzyme (made by diluting stock enzyme solutions in oxine–gelatin) are shown in Table 1. With increasing concentrations of enzyme but constant antibody–enzyme ratios, the plateau zone and neutralization slope were unaffected. The residual activity, however, is proportional to the initial enzyme titre.

Mixtures of normal and abnormal enzyme containing equal amounts of enzyme activity show a neutralization curve intermediate between the normal and the abnormal type. The results shown in Table 2 were obtained by neutralizing a mixture of 20 units of normal + 20 units of abnormal enzyme. In this case the neutralization slope of the mixture was about 245 neutralization units/ml. compared

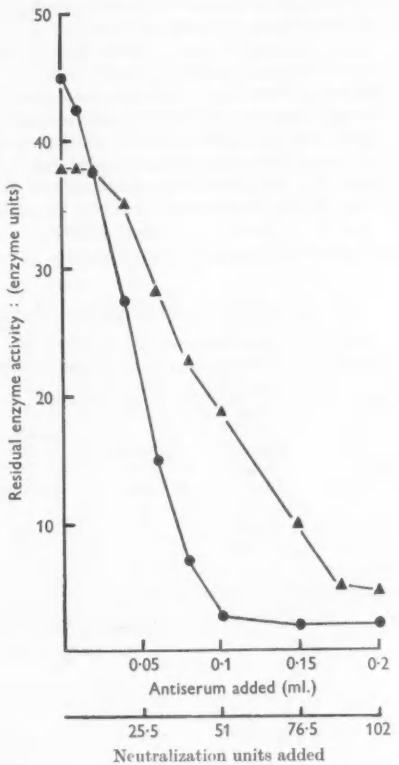


Fig. 5. Comparison of the neutralization characteristics of normal exopenicillinase (●) and that synthesized in the presence of *p*-fluorophenylalanine (▲).

with 420 neutralization units/ml. for normal and 115 neutralization units/ml. for abnormal enzyme. Although difficult to measure accurately, the plateau zone of the mixture was about three-quarters of that of unmixed abnormal enzyme of the same titre. The residual activity of mixtures of normal and abnormal enzyme was generally higher than that obtained with normal enzyme alone.

It follows from these experiments therefore that there is a *prima facie* case for believing that the enzyme synthesized in the presence of *p*-fluorophenylalanine reacts abnormally with antiserum prepared against pure normal exopenicillinase. There are, however, a number of alternative explanations which must first be excluded.

It is possible that some low-molecular-weight contaminant of the abnormal enzyme preparation not present, or present to a lesser extent, may interfere with the reaction between abnormal enzyme and antiserum. Salt is known to affect normal immunological precipitation reactions (see Boyd, 1956) but enzyme synthesized in the presence of *p*-fluorophenylalanine shows abnormal neutralization properties even after exhaustive dialysis against mM-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0.

Interference by some large-molecular-weight compound, unrelated to penicillinase, is excluded by studying the effect on the neutralization of normal penicillinase of exocellular-protein preparations obtained by growing uninduced *B. cereus* 569 in an identical medium to that used for production of the abnormal enzyme. Addition of undiluted culture filtrates, obtained in this way, to normal enzyme preparations had no effect on the subsequent neutralization properties of the enzyme. On the other hand, culture filtrates of *B. cereus* 569, which had been induced with 1 unit of benzylpenicillin/ml. in the presence of 50 μg. of *p*-fluorophenylalanine/ml., yielded enzyme indistinguishable, immunologically, from abnormal enzyme prepared from the constitutive strain (see also Pollock, 1956*a*). Further, mixtures of equal activities of normal and abnormal enzyme from induced cultures behaved on neutralization with antiserum essentially as similar preparations made from the constitutive strain. As the only exocellular material known to be elaborated by *B. cereus* in response to addition of penicillin is penicillinase, it seems inescapable that the material reacting atypically with normal anti-exopenicillinase serum is a penicillinase or penicillinases with immunological properties modified by growth in the presence of *p*-fluorophenylalanine. The involvement of the analogue is confirmed by the fact that phenylalanine added to a growth medium containing *p*-fluorophenylalanine completely protects the culture from inhibition (even if the phenylalanine concentration is only about one-fifth of that of the *p*-fluoro derivative) and that the penicillinase synthesized under these conditions is indistinguishable from normal.

Up to this point, experiments on the immunological properties of penicillinase synthesized in the presence of *p*-fluorophenylalanine were all carried out on enzyme obtained from the growth medium after incubation of cells for 2 hr. in the presence of

Table 1. *Neutralization characteristics of exopenicillinase preparations*

The plateau zone was estimated as described in the text (see also Fig. 4). 0 represents a value too small for satisfactory estimation; e.u., enzyme units; n.u., neutralization units.

Normal exopenicillinase				*p*-Fluorophenylalanine exopenicillinase			
Enzyme concn. (e.u./ml.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)	Enzyme concn. (e.u./ml.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
86	11±0·5	43	7·3	81	25±3·0	16·7	9·8
43	5±0·5	43	5·3	40·5	17±2·0	16·4	11·3
30·5	(1·5±0·5)	45	6·8	30·3	9±0·5	17·3	12·2
21·5	0	45	48	20·2	4±0·5	15·4	11·0

Table 2. *Neutralization characteristics of mixed exopenicillinase preparations*

For details see text; n.u., neutralization units.

	Normal exopenicillinase (42 units/ml.)	*p*-Fluorophenylalanine exopenicillinase (38 units/ml.)	Normal exopenicillinase (21 units/ml.) + *p*-fluorophenylalanine exopenicillinase (19 units/ml.)
Plateau zone (n.u.)	2·0±0.5	15±2·0	12±2
Neutralization slope (n.u./0·1 ml.)	42	17·5	24·5
Residual activity (% of initial activity)	7·2	9·8	11·5

the analogue. To study the immunological properties of enzyme found at various intervals after the addition of the analogue, a culture was incubated as described previously in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenylalanine/ml. and samples were removed at intervals for analysis. Table 3 shows the neutralization characteristics of the enzyme samples prepared in this way. The 'abnormality' of the enzyme (measured in terms of the width of the plateau zone and the neutralization slope) increases as incubation in the presence of *p*-fluorophenylalanine continues, reaching a maximum value after about 100–120 min. The relation of this finding to the increasing rate of *p*-fluorophenylalanine incorporation reported above has been investigated further in work described in the following paper (Richmond, 1960).

Neutralization of enzyme from canavanine-grown organisms. To test the neutralization characteristics of enzyme, formed in the presence of canavanine, organisms growing exponentially in 17AA(−Arg) medium were centrifuged down, washed in 0·9 % sodium chloride solution and resuspended to a density of about 0·2 mg. dry wt. of bacteria/ml. in 17AA(−Arg) medium containing 50 μg. of canavanine/ml. After incubation for 60 min. in this medium the organisms were removed by centrifuging and samples of exocellular enzyme prepared for titration with antiserum. Table 4 contains details of the neutralization characteristics of this enzyme preparation. As with exopenicillinase formed in the presence of *p*-fluorophenylalanine, the enzyme from canavanine-grown cells showed an increased width of plateau zone associated with a given amount of enzyme (measured in terms of activity). Under these conditions the plateau zone was equivalent to about 15 neutralization units/40 enzyme units. The residual enzyme activity was about 1·5 times the normal, but, unlike the enzyme from *p*-fluorophenylalanine-grown cells, the neutralization slope of the antiserum was about 85–90 % of that achieved with normal enzyme. Similar control experiments to those reported above showed that the abnormal neutralization characteristics of the enzyme formed in the presence of canavanine were due to a large-molecular-weight compound related to penicillinase. Neutralization of mixtures of equal amounts (on a basis of enzyme activity) of normal and abnormal enzyme showed similar characteristics to those reported previously; the width of the plateau zone was decreased by the presence of normal enzyme and the residual enzyme activity was related closely to the total initial enzyme titres. The changes observed in the neutralization slope during experiments of this kind were within the limits of experimental error, so it is not certain whether the addition of normal enzyme to enzyme formed in the presence of canavanine resulted in changes in the neutralization phase of the curve.

The neutralization characteristics of abnormal enzyme were examined at intervals after the addi-

Table 3. *Neutralization characteristics of exopenicillinase harvested at intervals after addition of* p-*fluorophenylalanine*

0 represents a value too low to measure accurately; e.u., enzyme units; n.u., neutralization units.

Time after addition of analogue (min.)	Enzyme titre (e.u.)	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
20	15	0	44	7·5
40	30	2±0·5	39	9·3
60	30	4±0·5	32	8·4
90	30	6±0·5	21	8·8
120	30	8±1·0	16·5	11·2
180	30	7±1·0	17·5	9·8
Normal enzyme	30	1·0±0·5	46·5	6·8

Table 4. *Neutralization characteristics of exopenicillinase synthesized in the presence of canavanine*

For details see text; e.u., enzyme units; n.u., neutralization units.

Exopenicillinase concn. (e.u./ml.)				
Normal enzyme	Canavanine-grown enzyme	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial activity)
—	40	16±2·0	33	10
—	30	9±1·0	33	13·2
—	20	5±0·5	34	11·3
30	—	2·0±0·5	37	6·8

tion of canavanine. The size of the plateau zone increased for only about 75 min. after the addition of the analogue, reaching a maximum value of about 18 neutralization units/40 enzyme units; the neutralization slope never fell below 85 % of the normal.

DISCUSSION

Growth of *B. cereus* in medium containing the amino acid analogues canavanine or *p*-fluorophenylalanine leads to the formation of material which reacts atypically with anti-exopenicillinase γ-globulin. As this material is formed by the inducible strain only after induction with penicillin, it is virtually certain that it must be a molecule related to penicillinase, rather than some non-specific factor resulting from the metabolic imbalance caused by addition of the analogues. As *p*-fluorophenylalanine is incorporated into the protein fraction of *B. cereus* 569/H grown in 17AA(−Phe) medium, and since Munier & Cohen (1956, 1959) and Cohen & Munier (1959) have shown that this analogue can replace phenylalanine in the protein fraction of cells grown in a glucose–ammonia–salts medium containing *p*-fluorophenylalanine, it seems possible that the material reacting atypically with anti-exopenicillinase γ-globulin consists of one or a number of abnormal penicillinases containing *p*-fluorophenylalanine in place of phenylalanine. Canavanine can be shown to be incorporated into the protein of *B. cereus* 569/H under suitable conditions and this could lead to the formation of abnormal penicillinase(s) containing canavanine in place of arginine.

Superficially, the abnormal characteristics of the enzymes formed in the presence of either analogue are similar. In both cases the main abnormality is the appearance of the plateau zone. This change is difficult to interpret. The neutralization of a preparation of normal exopenicillinase probably involves a reaction between a single homogeneous antigen (the exopenicillinase) and a heterogeneous collection of γ-globulin molecules with varying efficacy in the neutralization of the enzyme. Neutralization of abnormal enzyme is more complicated, for there is good reason to believe (see Richmond, 1960) that a heterogeneous collection of penicillinase molecules is formed in the presence of *p*-fluorophenylalanine and that these may react to a varying extent with the heterogeneous collection of antibody molecules. A reaction of this kind could readily lead to a modified neutralization curve.

It is conceivable that the presence of the plateau zone could imply that the abnormal enzyme preparations are mixtures containing normal enzyme together with a proportion of abnormal molecules, which are devoid of enzyme activity but which have a higher affinity than normal enzyme for the antiserum. Normal enzyme, however, which is known to be a single protein (Kogut *et al.* 1956), shows a small plateau zone and this is probably just an expression of impaired neutralization at low antibody–enzyme (antigen) ratios. Although a plateau zone has not been specifically reported in connexion with the neutralization of other enzymes by antisera, it is frequently encountered in immunological precipitation reactions (see Boyd, 1956) and may be detected in the enzyme neutralization curves published by several authors (e.g. Campbell & Fourt, 1939; Macfarlane & Knight, 1941; Adams, 1942; Krebs & Najjar, 1948; Henion, Mansour & Bueding, 1955). Under these circumstances therefore it seems more likely that the increased width of the plateau zone is due to the presence of abnormal penicillinase(s) with impaired specific activity and ability to be neutralized by normal antiserum. The lowered differential rate of penicillinase synthesis occurring after the addition of the analogues (see Fig. 2), and the lowered neutralization slope exhibited by the antisera, could be due to the formation of penicillinase-like proteins with lowered specific enzyme activity.

The nature of the material reacting atypically with anti-exopenicillinase γ-globulin is discussed further at the end of the next paper (Richmond, 1960).

SUMMARY

1. Addition of canavanine to cultures of *Bacillus cereus* growing exponentially in fully synthetic amino acid media, lacking arginine, leads to complete inhibition of growth and exopenicillinase formation after incubation for about 90 min. in the presence of the analogue. The differential rate of exopenicillinase formation is, however, markedly inhibited before growth ceases completely.

2. Addition of *p*-fluorophenylalanine to cultures growing exponentially in a fully synthetic medium lacking phenylalanine leads to impaired growth which nevertheless continues in an approximately linear manner for 4–5 hr. During this period the differential rate of exopenicillinase synthesis is increasingly inhibited, falling to about half normal after 90 min. incubation in the presence of the analogue.

3. Comparison of the immunological neutralization of exopenicillinase formed in the presence of canavanine or *p*-fluorophenylalanine with normal exopenicillinase suggests that the abnormal enzyme preparations may have impaired specific enzyme activity and lowered efficiency of reaction with specific anti-exopenicillinase serum.

4. The appearance of the abnormal material may be induced in the inducible (569) strain of

B. cereus under the conditions necessary for induction of penicillinase in normal cultures.

5. The immunological 'abnormality' of exopenicillinase preparations obtained from cultures increases as incubation in the presence of the amino acid analogues is prolonged.

REFERENCES

Adams, M. H. (1942). *J. exp. Med.* **76**, 175.
Boyd, W. C. (1956). *Fundamentals of Immunology*, 2nd ed. New York: Interscience Publishers Inc.
Campbell, D. H. & Fourt, L. J. (1939). *J. biol. Chem.* **129**, 385.
Cohen, G. W. & Munier, R. (1959). *Biochim. biophys. Acta*, **31**, 347.
Cowie, D. B., Cohen, G. N., Bolton, E. & de Robichon-Szulmajster, H. (1959). *Biochim. biophys. Acta*, **34**, 39.
Henion, W. F., Mansour, T. E. & Bueding, E. (1955). *Exp. Parasit.* **4**, 40.
Kantorowicz, O. (1951). *J. gen. Microbiol.* **5**, 276.
Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
Krebs, E. G. & Najjar, V. A. (1948). *J. exp. Med.* **88**, 599.
Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Munier, R. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
Munier, R. & Cohen, G. N. (1959). *Biochim. biophys. Acta*, **31**, 378.
Pollock, M. R. (1952). *Brit. J. exp. Path.* **33**, 587.
Pollock, M. R. (1956*a*). *J. gen. Microbiol.* **14**, 90.
Pollock, M. R. (1956*b*). *J. gen. Microbiol.* **15**, 156.
Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
Pollock, M. R. & Perret, C. J. (1951). *Brit. J. exp. Path.* **32**, 387.
Richmond, M. H. (1959*a*). *Biochem. J.* **73**, 155.
Richmond, M. H. (1959*b*). *Biochem. J.* **73**, 261.
Richmond, M. H. (1960). *Biochem. J.* **77**, 121.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). In *Studies of biosynthesis in Escherichia coli*. Washington: Carnegie Institution of Washington (Publication No. 607).
Urbá, R. C. (1959). *Biochem. J.* **71**, 513.

Biochem. J. (1960) **77**, 121

Incorporation of DL-β-(*p*-Fluorophenyl)[β-^{14}C]alanine into Exopenicillinase by *Bacillus cereus* 569/H

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Experiments reported in the preceding paper (Richmond, 1960) have shown that addition of *p*-fluorophenylalanine to cultures of *Bacillus cereus* 569/H (constitutive strain) led to the formation of material which has the properties of immunologically abnormal penicillinase(s). Munier & Cohen (1956, 1959) and Cohen & Munier (1959) have reported that *p*-fluorophenylalanine replaces phenylalanine but not tyrosine in the total cell-protein fraction of *Escherichia coli* and Vaughan & Steinberg (1958) have detected [^{3}H]-*p*-fluorophenylalanine in purified ovalbumin and lysozyme isolated from hen-oviduct preparations that had been incubated with tritiated *p*-fluorophenylalanine. Kruh & Rosa (1959) incubated rabbit reticulocytes *in vitro* with [^{14}C]-*p*-fluorophenylalanine and detected the analogue in haemoglobin prepared from the cells. In view of these findings, it was decided to measure the incorporation of *p*-fluorophenylalanine into the exopenicillinase fraction of *B. cereus* 569/H to try to relate the degree of incorporation of the analogue with the abnormal immunological properties of the enzyme (see Richmond, 1960).

Unfortunately the one feasible way of isolating pure exopenicillinase in the quantity available from these experiments is by precipitation with specific antiserum (Pollock & Kramer, 1958). This immediately limits the types of exopenicillinase molecules studied in this work to those precipitable with antiserum, and it is proposed to call this group of proteins the 'exopenicillinase-type proteins'. The reasons for believing that the exopenicillinase-type proteins are, in fact, related to exopenicillinase are given by Richmond (1960).

A further complication of this method of isolation of the exopenicillinase-type protein is that it is impossible to obtain an accurate measure of the specific *p*-fluorophenylalanine incorporation (μg. of *p*-fluorophenylalanine/μg. of exopenicillinase-type protein). Further, as the specific enzyme activity (enzyme units/μg. of exopenicillinase-type protein) may be impaired under these conditions (Richmond, 1960), enzyme activity cannot be used as an

accurate measure of the quantity of enzyme protein present in the preparations. It has, however, proved possible to measure the quantity of protein indirectly by following the incorporation of L-[^{14}C]valine. The differential rate of incorporation of this amino acid into exopenicillinase-type protein preparations was unaffected by the presence of *p*-fluorophenylalanine and the total amount of radioactivity incorporated was a measure of the L-valine content of the exopenicillinase-type protein. This, in turn, was related to total quantity of protein in the exopenicillinase-type protein preparations and allowed the calculation of specific enzyme activities. In parallel experiments it was possible to measure the incorporation of *p*-fluorophenyl[β-^{14}C]alanine into exopenicillinase-type protein and associate this with the effect of the analogue on the specific enzyme activity (enzyme units/μg. of exopenicillinase-type protein). Further experiments, in turn, associated these changes with the abnormal immunological properties of exopenicillinase-type proteins.

Munier & Cohen (1956, 1959) have reported that *p*-fluorophenylalanine completely replaces phenylalanine in proteins synthesized in the presence of the analogue. As normal exopenicillinase from *B. cereus* 569 (inducible strain) and 569/H (constitutive mutant strain) contains about seven phenylalanine residues, it would be interesting to determine the degree of replacement of phenylalanine in exopenicillinase-type proteins by the *p*-fluoro derivative. For technical reasons (see below) it is not possible to measure this by the effect of *p*-fluorophenylalanine on [^{14}C]phenylalanine incorporation into exopenicillinase-type proteins and therefore the degree of replacement must be estimated indirectly. There is no increase in the phenylalanine content of the total cell protein or the total exocellular protein after incubation for about 60 min. in the presence of the analogue. Subsequently the *p*-fluoro derivative replaced an increasing number of the phenylalanine residues. As the relative proportion of *p*-fluorophenylalanine appearing in a number of protein fractions from the organisms (including exopenicillinase-type proteins) is constant under these conditions it is likely that most of the phenylalanine in the exopenicillinase-type proteins has been replaced by the *p*-fluoro derivative. The implications of these findings are discussed in the light of the experiments outlined above and the results reported by Richmond (1960).

MATERIALS AND METHODS

Organisms and media. The organisms used in these studies, together with their preparation for experimental purposes and the composition of casein-hydrolysate medium, 18AA medium and 18AA(−Phe) medium have been described by Richmond (1960). For specific purposes, 18AA medium was modified by omitting valine [17AA(−Val) medium] or phenylalanine and valine [16AA(−Phe−Val) medium].

Amino acids. The *p*-(fluorophenyl)[β-^{14}C]alanine was obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette (Seine et Oise), France, at a specific activity of 2·35 μc/μmole. The L-[^{14}C]valine was generally labelled and was obtained from The Radiochemical Centre, Amersham, Bucks, at a specific activity of 4·0 μc/μmole.

Anti-exopenicillinase. Neutralization experiments were carried out with rabbit anti-exopenicillinase γ-globulin no. 362; it had a titre of about 10^4 neutralization units/ml. For experiments involving precipitation of exopenicillinase preparations (see Results section) a preparation made by Pollock & Kramer (1958) was used; it had a titre of $1{\cdot}2 \times 10^5$ neutralization units/ml. Both anti-penicillinase preparations were diluted in 0·9% sodium chloride solution containing 0·8 mM-oxine to a titre of about 450 neutralization units/ml. and stored at −20°.

Chemical fractionation. The following fractionation procedure was used to isolate the internal 'pool', lipid, ethanol-soluble protein, total nucleic acid and protein + cell-wall fractions (see McQuillen & Roberts, 1954). Samples containing about 1·0–2·0 mg. dry wt. of organisms were centrifuged at 3000 *g*, for 15 min. and the supernatant growth medium was discarded.

(1) Internal 'pool'. The organisms were resuspended in 5% (w/v) trichloroacetic acid and stored at 2° for 2–3 hr. The supernatant obtained after centrifuging was pooled with a similar supernatant obtained by a second extraction and constituted the internal 'pool' fraction.

(2) Lipid and ethanol-soluble protein. The insoluble residue obtained from (1) was extracted twice with aqueous ethanol (75%, v/v) at 75°. The extracts obtained on centrifuging were pooled and constituted the mixed lipid + ethanol-soluble protein fraction.

(2*a*) The ethanol-soluble protein was separated from the lipid by mixing the extract obtained in (2) with 3 vol. of ether + 3 vol. of water. After thorough mixing the ether layer was removed and the water phase, which contains the ethanol-soluble protein fraction, was dried *in vacuo*.

(3) Total nucleic acids. The cell pad obtained in (2) was extracted three times for 20 min. with 5% trichloroacetic acid at 95° and the extracts were combined. More than 90% of the total nucleic acid in the organisms may be extracted in this way.

(4) The residue from (3) constituted the protein + cell-wall fraction. It was washed successively with aqueous ethanol (75%, v/v) and ether and dried in a stream of air. (Yield, 45–60% of the original dry wt. of the organisms.)

Hydrolysis. Hydrolysis to liberate peptides from the protein + cell-wall fraction was carried out in 12N-HCl at 35°. Samples removed at intervals and analysed by chromatography and ionophoresis showed that hydrolysis for 36 hr. gave the most satisfactory yield of peptide material. To liberate free amino acids, the protein + cell-wall fraction was hydrolysed for 16 hr. in 6N-HCl at 105° in a sealed tube. The HCl was removed *in vacuo*.

Ionophoresis. Peptides were separated in collidine–acetic acid–water (0·7:0·3:100, by vol.), pH 7·0 (Newton & Abraham, 1954). The neutral and acidic bands obtained at this pH were eluted in water and run again in 2·0M-acetic acid (pH 2·4). Ionophoresis was carried out in an apparatus

of the type described by Kunkel (1954), with a potential gradient of about 15 v/cm.

Chromatography. Free amino acids obtained by hydrolysis of the protein + cell-wall fraction were separated by descending chromatography in butanol–acetic acid–water (60:10:26, by vol.). For quantitative determination of the relative quantities of tyrosine, phenylalanine and leucine + isoleucine (+*p*-fluorophenylalanine, if present) hydrolysates of the protein + cell-wall fraction were transferred quantitatively to Whatman no. 3 paper as 5 cm. bands at the origin. The chromatograms were developed for 19–20 hr. and the location and quantity of the amino acids present determined as described by Mandelstam & Rogers (1959). The $R_{phenylalanine}$ values of the tyrosine, valine + methionine and leucine + isoleucine + *p*-fluorophenylalanine spots were about 0·68, 0·87 and 1·16 respectively. The quantity of *p*-fluorophenyl[β-^{14}C]alanine present was determined by estimating the radioactivity present in a representative sample removed from the eluate containing leucine + isoleucine + *p*-fluorophenylalanine after development with ninhydrin. Separate experiments showed that known quantities of *p*-fluorophenyl[β-^{14}C]alanine treated on paper with ninhydrin were not appreciably volatile unless the papers were moist and heated to temperatures greater than 50°.

Isolation and estimation of total exocellular protein. Samples of about 10–20 ml. were removed from cultures and centrifuged at 3000 *g*, for 30 min. The supernatant growth medium was decanted into tubes and centrifuged again under the same conditions for a further 30 min. The supernatant obtained in this way was passed through an Oxoid membrane filter and mixed with sufficient 50% (w/v) trichloroacetic acid to give a final concentration of 5%. After standing overnight at 2° the protein precipitates were centrifuged at 3000 *g* for 30 min.; the centrifuge tubes were then turned in the centrifuge cups through 180° and the precipitates resedimented at 3000 *g* for a further 30 min. The supernatants were decanted and the protein precipitates estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Suitable dilutions of crystalline bovine-serum albumin (Armour Laboratories Ltd.) were used as standards. When it was not required to estimate the total amount of exocellular protein (e.g. when radioactive determinations only were required), 100 μg. of serum albumin/ml. was added to the filtrate before precipitation. The 'carrier' greatly facilitated handling of the precipitates and the centrifuging time could be reduced to one period of 20 min. at 3000 *g*.

Radioactivity determinations. Samples were prepared for counting as described by Richmond (1959). Counting was continued until more than 10^3 counts above background had been registered, and the samples were assumed to be infinitely thin unless they contained more than 0·5 mg. dry wt. of material/cm.^{2} A sample of [^{14}C]valine containing 1·0 μmc registered 193 counts/min. in the equipment used.

Determination of differential rates. Unless otherwise stated, all differential rates have been measured as described by Richmond (1959). The growth of cultures was followed turbidimetrically and the culture density determined by reference to a standard curve. When incubation in the presence of *p*-fluorophenylalanine was continued for long periods, it was found experimentally that the standard curve no longer gave a true estimate of dry weight of organisms present in these cultures. This was due to the lower scattering caused by the increased size of organisms obtained under these conditions (see Mitchell, 1949). In practice, however, all differential rates have been measured on the basis of the standard curve; the resulting apparent stimulation in differential rate of the inhibited culture, due to this procedure, is only about 10% above normal after incubation for 2 hr. in the presence of *p*-fluorophenylalanine and increases of this order have been discounted.

Purification of radioactive exopenicillinase and exopenicillinase-type protein. Both exopenicillinase and exopenicillinase-type protein were purified as described by Pollock & Kramer (1958) except that non-radioactive 'carrier' exopenicillinase was not added because it has been shown that exopenicillinase synthesized in the presence of *p*-fluorophenylalanine has abnormal neutralization characteristics (Richmond, 1960) and may not therefore be a true carrier.

The validity of this method for purifying the exopenicillinase preparation depends upon the following considerations.

(*a*) All the enzyme proteins comprising the abnormal preparation react with glass powder. To test this point, crude preparations (see Richmond, 1960) of normal or abnormal exopenicillinase were adsorbed by step-wise addition of increasing quantities of glass powder. Fig. 1 shows that about three times the amount of glass powder required for the normal preparation had to be added for maximum adsorption of the abnormal penicillinase. The total amount of enzyme adsorbed was, however, approximately the same in both cases (90%). The elution patterns of the normal and abnormal enzyme preparations were similar in these experiments; the immunological characteristics of the eluted enzyme, the enzyme not adsorbed on glass and the enzyme initially added to the experiment were approximately the same (see Table 1). It appears therefore that the 'abnormal' enzyme behaves in a manner sufficiently homogeneous to allow use of adsorption on to glass powder as a step in the purification, and there is no

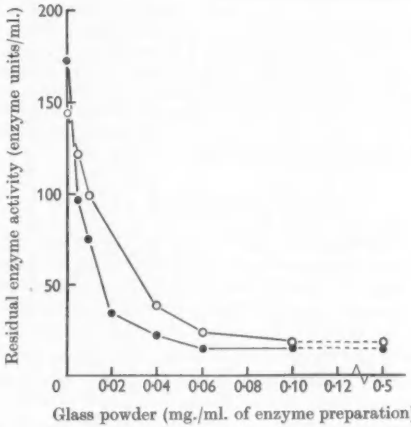

Fig. 1. Adsorption of normal exopenicillinase and exopenicillinase-type protein on to glass powder. ●, Normal enzyme; ○, exopenicillinase-type protein

Table 1. *Neutralization characteristics at various stages of purification of exopenicillinase-type protein preparations from* Bacillus cereus 569/H *grown in the presence of* p-*fluorophenylalanine*

Normal enzyme was purified as described by Pollock & Kramer (1958). The abnormal enzyme was purified in a similar way except that three times the quantity of glass powder was used per unit of enzyme activity (see text and fig. 1). e.u., Enzyme units; n.u., neutralization units.

Enzyme preparation	Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (% of initial enzyme activity)	Initial enzyme activity (e.u./ml.)
Crude normal	4±1	43	5·7	35
Crude abnormal	17±2	18·3	9·3	27
Residue after maximum glass adsorption	18±2	16	9·8	17
Eluted enzyme	15±2	19·5	9·5	43

evidence for preferential elution of any one fraction of the mixed exopenicillinase-type molecules. In addition, it was not possible to detect the non-adsorption of any cross-reacting protein devoid of enzyme activity.

(*b*) Addition of 1·5 equivalent quantities of antiserum completely precipitated (> 90%) normal exopenicillinase preparations (Pollock & Kramer, 1958). To check that the same was true of abnormal enzyme preparations, samples were titrated with antiserum to estimate the equivalence point and then treated with 1·5 equivalence doses of antiserum at concentrations of enzyme at which precipitation occurred. In all cases 85% or more of the enzyme activity was precipitated. The equivalence point of abnormal enzyme treated in this way was frequently two to three times as high as that of the normal enzyme. In no case was there any sign of solubility of enzyme–antiserum precipitates in excess of antibody (cf. Boyd, 1956). With this method of purification there is a high inherent risk of contamination of the enzyme–antiserum precipitates with non-specific radioactivity. The level of contamination was controlled in parallel experiments by incubating a culture of the uninduced inducible strain (569) under identical conditions to the experimental culture of the constitutive strain (569/H) and then adding non-radioactive 'carrier' exopenicillinase at the end of the experiment. Isolation of the exopenicillinase from such cultures, followed by radioactivity determinations, controlled the level of contamination both by high-molecular-weight materials precipitated with the anti-exopenicillinase–enzyme precipitates and by non-specific adsorption of low-molecular-weight radioactive material on to the precipitates [see Pollock & Kramer (1958) for detailed discussion of these points].

RESULTS

Preliminary experiments on the effect of *p*-fluorophenylalanine on exopenicillinase formation showed that an independent method of measuring the protein content of exopenicillinase-type protein was necessary before any deductions could be made about the effect of the analogue on specific enzyme activity. Because preliminary experiments were hopeful, but despite earlier evidence to the contrary (Halvorson & Spiegelman, 1952), it was decided to see whether the incorporation of amino acids other than phenylalanine into exopenicillinase could be used as a measure of exopenicillinase

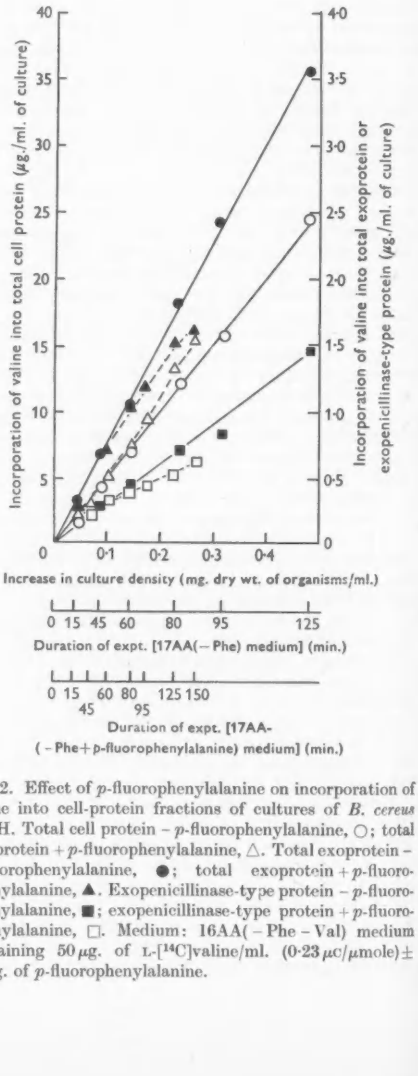
Incorporation of valine into total cell protein (μg./ml. of culture)
Incorporation of valine into total exoprotein or exopenicillinase-type protein (μg./ml. of culture)
Increase in culture density (mg. dry wt. of organisms/ml.)
Duration of expt. [17AA(− Phe) medium] (min.)
Duration of expt. [17AA- (− Phe+p-fluorophenylalanine) medium] (min.)

Fig. 2. Effect of *p*-fluorophenylalanine on incorporation of valine into cell-protein fractions of cultures of *B. cereus* 569/H. Total cell protein − *p*-fluorophenylalanine, ○; total cell protein + *p*-fluorophenylalanine, △. Total exoprotein − *p*-fluorophenylalanine, ●; total exoprotein + *p*-fluorophenylalanine, ▲. Exopenicillinase-type protein − *p*-fluorophenylalanine, ■; exopenicillinase-type protein + *p*-fluorophenylalanine, □. Medium: 16AA(− Phe − Val) medium containing 50 μg. of L-[^{14}C]valine/ml. (0·23 μc/μmole)± 50 μg. of *p*-fluorophenylalanine.

protein synthesis. To test this possibility, the effect of *p*-fluorophenylalanine was studied on the incorporation of [^{14}C]valine into the total cell protein, total exocellular protein and exopenicillinase-type protein of cultures of *B. cereus* 569/H incubated in 16AA(−Phe−Val) medium ± 50 μg. of *p*-fluorophenylalanine/ml. (Fig. 2). A culture of the inducible strain (569) was incubated under identical conditions (i.e. in the absence of inducer) and used as a control (see Materials and Methods section). The differential rate of [^{14}C]valine incorporation into the total cell-protein fraction was unaffected by the analogue (actually about 10% stimulation, but see Materials and Methods section) the incorporation both into the total exocellular protein and exopenicillinase-type protein appeared slightly depressed. The depression in differential rate of valine incorporation into the exocellular proteins could either have been due to a fall in the total amount of exocellular protein synthesized per unit growth or to a specific effect of the analogue on valine incorporation. To decide between these possibilities, a culture was incubated in 16AA(−Phe−Val) medium containing *p*-fluorophenylalanine + [^{14}C]valine and the specific radioactivities (counts/min./μg. of protein) of the total cell protein and total exocellular protein were determined in the absence of carrier. Table 2 shows that the specific radioactivity of the protein fractions was approximately the same, despite a fall of about 10% in the differential rate of total exocellular-protein synthesis. This implies that *p*-fluorophenylalanine has no specific effect upon the valine content of either protein fractions and it is reasonably certain therefore that it has no effect upon the incorporation of valine into the exopenicillinase-type protein.

In practice it was possible to use the appearance of ^{14}C from [^{14}C]valine in exopenicillinase-type protein as a measure of the valine content of that fraction. This depends upon the following conditions, which were found to be fulfilled:

(1) *B. cereus* requires an external supply of valine for normal growth and enzyme formation (M. H. Richmond, unpublished work).

(2) When organisms are grown in 17AA(−Val) medium containing [^{14}C]valine ± *p*-fluorophenylalanine, all but about 5% of the radioactivity in the protein + cell-wall fraction is present as valine.

(3) Protein breakdown in this strain is about 8–10%/hr. in non-growing cultures. The rate of breakdown in growing cultures is usually markedly less (see Pollock & Kramer, 1958; Urbá, 1959). This means that at a culture density of 0·3 mg. dry wt. of organisms/ml. the supply of valine by protein breakdown is at the most 0·05–0·08 μg. of L-valine/ml./hr. This is approx. 0·1% of the external valine concentration at the beginning of

Table 2. *Specific radioactivity of total cell- and exocellular-protein fractions from cultures of* Bacillus cereus 569/H *incubated in the presence of* p-*fluorophenyladanine and* L-[^{14}C]*valine*

Organisms growing exponentially in 17AA(−Phe) medium were harvested and resuspended to a density of about 10 μg. dry wt. of bacteria/ml. in 16AA(−Phe−Val) medium containing 50 μg. of L-[^{14}C]valine/ml. (0·34 μc/μmole). After growth for 3 hr. in this medium *p*-fluorophenylalanine (50 μg./ml.) was added to the culture and samples of bacteria taken at intervals were treated to determine the specific radioactivity of the protein + cell wall and the total exocellular-protein fractions. Values in parentheses are approximate because of the inaccuracy of the protein estimations.

Interval from addition of analogue (hr.)	Interval from addition of tracer (hr.)	Culture density (mg./ml.)	Protein content of organisms (μg. of protein/mg. dry wt. of organisms)	Specific radioactivity in protein + cell-wall fraction (counts/min./μg. of protein)	Total exocellular protein (μg. of protein/mg. dry wt. of organisms)	Specific radioactivity of exoprotein (counts/min./μg. of protein)
0	3·0	0·09	395	26·7	10·3	(20)
0·5	3·5	0·15	415	25·4	11·3	(20)
1·0	4·0	0·23	420	26·1	10·4	21
1·5	4·5	0·32	395	28·0	10·6	21
2·0	5·0	0·405	375	27·6	9·8	23
3·0	6·0	0·52	415	26·8	9·3	24

Table 3. *Valine content of normal exopenicillinase synthesized by* Bacillus cereus *569/H in* 17AA(−Val) *medium with* L-[^{14}C]*valine*

A culture growing exponentially in 18AA medium was harvested and the organisms were transferred to 17AA(−Val) medium containing 50 μg. of L-[^{14}C]-valine/ml. (0·23 μc/μmole). Samples taken at intervals were treated to yield purified exopenicillinase as described in the text. e.u., Enzyme units.

Interval after addition of tracer (min.)	Culture density (mg./ml.)	Total amount of crude exopenicillinase (e.u.)	Total amount of purified exopenicillinase (e.u.)	Recovery (%)	Exopenicillinase protein equivalent of purified enzyme* (μg.)	Specific radioactivity of purified exopenicillinase (μmc/10^3 e.u.)	Total L-valine content of exopenicillinase (μg.)	Valine content of purified exopenicillinase (%, by wt.)
0	0·15	—	—	—	—	—	—	—
0	0·23	4 950	1 640	33	5·2	0·32	0·27	5·1
40	0·33	15 200	6 200	41	19·8	0·28	0·87	4·4
60	0·49	32 700	9 450	29	30·2	0·26	1·27	4·2
90	0·62	52 900	19 650	38	62·1	0·30	3·0	4·8
120							Av.	4·6

* 1 μg. of crystalline exopenicillinase ≡ 312 enzyme units.

the experiment and about 0·5 % of that at the end. These quantities will not reduce, significantly, the specific activity (μc/μmole) of the [^{14}C]valine incorporated.

(4) The 'pool' concentration of [^{12}C]valine at the beginning of the experiment does not dilute the exogenous [^{14}C]valine to an appreciable extent. This can be seen from the result shown in Fig. 2, in which the differential rate of [^{14}C]valine incorporation into the protein + cell wall is constant throughout the experiment and extrapolates through the origin.

Strong additional support for this method of estimating the valine content of a protein fraction is given by the experiments designed to measure the specific valine content of normal purified exopenicillinase. A culture growing exponentially in 18AA medium was centrifuged, the organisms were washed in 17AA(−Val) medium and then resuspended [to a density of about 0·1 mg. dry wt. of bacteria/ml.] in 17AA(−Val) medium containing L-[^{14}C]valine. The culture was incubated under normal conditions and samples, removed at intervals, were treated to yield the purified exopenicillinase fraction. The specific radioactivity of the exopenicillinase on an activity basis (μc/units of enzyme activity) was found to be approximately constant throughout the experiment (see Table 3). As the specific enzyme activity of crystalline exopenicillinase from this strain is known (Kogut, Pollock & Tridgell, 1956), it is possible to calculate the specific valine content of exopenicillinase. Table 3 shows that this value is approximately constant at an average of 4·6 %, which agrees reasonably well with the mean value of about 5·0 % obtained by chemical methods (S. Jacobs, personal communication). As *p*-fluorophenylalanine has no effect upon the differential rate of total cell protein, total exocellular protein, nor upon exopenicillinase-type protein synthesis, and as no effect of the analogue can be detected on the incorporation of [^{14}C]valine into any protein fraction investigated, it follows that the incorporation of [^{14}C]valine into exopenicillinase-type protein in these experiments provides a measure of the protein content of that fraction.

Incorporation of β-(p-*fluorophenyl*)-[β-^{14}C]*alanine into cell fractions*

A culture growing exponentially in 18AA medium was centrifuged and the organisms, after washing in 17AA(−Phe) medium, were resuspended to a density of about 0·1 mg. dry wt. of bacteria/ml. in 17AA(−Phe) medium containing 50 μg. of *p*-fluorophenyl[β-^{14}C]alanine/ml. (0·17 μc/μmole) and incubated under standard conditions. Samples removed at intervals were fractionated as

described in the Materials and Methods section to yield the internal 'pool', lipid, ethanol-soluble protein, total nucleic acid and protein + cell-wall fractions. The distribution of radioactivity in these fractions after incubation for 2 hr. in the presence of the analogue is shown in Table 4. More than 70% of the radioactivity was found in the protein + cell-wall fraction. Isolation of the bacterial cell walls from organisms grown under similar conditions showed that the amount of *p*-fluorophenylalanine incorporated was about 5% of that incorporated into an equal weight of protein + cell-wall fraction. Hydrolysis of the protein + cell-wall fraction obtained under these conditions in 12N-HCl at 35° for 36 hr. followed by ionophoresis of the product in collidine–acetate buffer, pH 7·0 (Newton & Abraham, 1954), showed the presence of radioactive material in the neutral and acidic bands of peptides obtained by this procedure. Further separation of the neutral peptides by ionophoresis in 2·0M-acetic acid at pH 2·4 showed the presence of a number of radioactive peptide components. It seemed very likely therefore that the incorporation of *p*-fluorophenylalanine into the protein of *B. cereus* entailed the formation of peptide bonds. Hydrolysis of the protein + cell-wall fraction to liberate free amino acids showed, on subsequent chromatography, only one radioactive

Table 4. *Distribution of* p-*fluorophenyl*[β-^{14}C]-*alanine in cell fractions of* Bacillus cereus 569/H *grown in the presence of the analogue*

	Cell fraction	Percentage of total radioactivity incorporated
1	Internal 'pool'	0*
2	Mixed lipid and ethanol-soluble protein	7
2*a*	Lipid (ether-soluble fraction)	2
2*b*	Ethanol-soluble protein (water-soluble fraction)	5
3	Total nucleic acid	5
4	Protein + cell wall	74
	Recovery (%)	93

* Pool concentration of *p*-fluorophenylalanine is very low; the maximum level detected corresponded to 0·2 μ-mole/g. dry wt. of organisms.

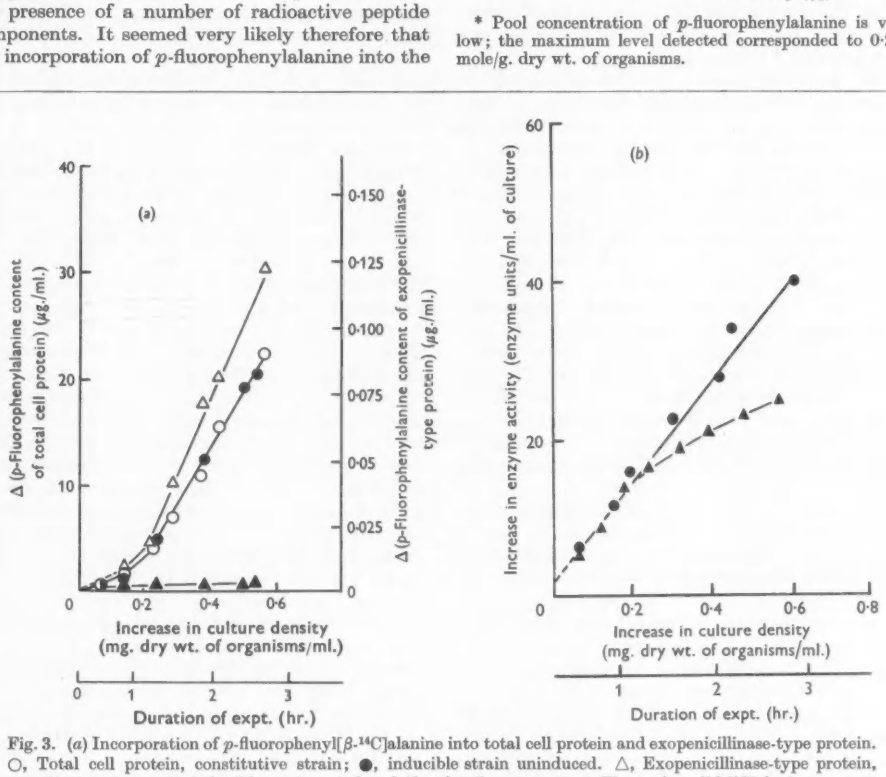
(a)
Δ (p-Fluorophenylalanine content of total cell protein) (μg./ml.)
Δ (p-Fluorophenylalanine content of exopenicillinase-type protein) (μg./ml.)
Increase in culture density (mg. dry wt. of organisms/ml.)
Duration of expt. (hr.)
(b)
Increase in enzyme activity (enzyme units/ml. of culture)
Increase in culture density (mg. dry wt. of organisms/ml.)
Duration of expt. (hr.)

Fig. 3. (*a*) Incorporation of *p*-fluorophenyl[β-^{14}C]alanine into total cell protein and exopenicillinase-type protein. ○, Total cell protein, constitutive strain; ●, inducible strain uninduced. △, Exopenicillinase-type protein, constitutive strain; ▲, inducible strain uninduced (for details see text). *p*-Fluorophenyl[β-^{14}C]alanine present was 50 μg./ml. (0·34 μc/μmole). (*b*) Effect of *p*-fluorophenylalanine on differential rate of appearance of exopenicillinase activity. ●, 17AA(−Phe) medium; ▲, 17AA(−Phe + *p*-fluorophenylalanine) medium.

spot corresponding to marker *p*-fluorophenylalanine. The specific radioactivity (μc/μmole) of the material isolated in this way was the same as that of the *p*-fluorophenylalanine added to the growth medium. It follows therefore that the radioactivity present in the protein + cell-wall fraction may be used as a direct measure of the amount of *p*-fluorophenylalanine incorporated into that fraction.

Incorporation of p-*fluorophenyl*[β-^{14}C]*alanine into total cell protein and exopenicillinase-type protein*

A culture was incubated in 17AA(−Phe) medium containing *p*-fluorophenyl[β-^{14}C]alanine and samples, removed at intervals, were treated to yield the total cell-protein and exopenicillinase-type protein fractions. Fig. 3 (*a*) shows that the differential rate of incorporation of fluorophenylalanine into these fractions of the constitutive strain increases for about 1 hr. before becoming approximately constant. Similar increases are found to occur in the uninduced inducible strain except that no appreciable activity can be detected in the isolated exopenicillinase-type protein fractions. Fig. 3 (*b*) shows the differential rate of appearance of exopenicillinase (measured in terms of activity) in the same experiment. Comparison of the differential rate of *p*-fluorophenylalanine incorporation into the exopenicillinase-type protein fraction with the change in rate of appearance of exopenicillinase activity shows that the increase in rate of analogue incorporation is associated with a fall in enzyme activity in the culture. It is not possible, from these experiments, however, to determine the increase in exopenicillinase-type protein associated with these changes. Further, although the foregoing experiments show that *p*-fluorophenylalanine is incorporated into the total protein and exopenicillinase fractions of the organisms, they cannot show whether the unnatural amino acid is incorporated in place of phenylalanine, nor the extent of the replacement, if any.

As a result of the experiments reported in the Results section it is possible to use the net incorporation of [^{14}C]valine into the exopenicillinase-type protein as a measure of total protein content of that fraction. Further, the net *p*-fluorophenylalanine content of the exopenicillinase-type protein may be measured by following the incorporation of *p*-fluorophenyl[β-^{14}C]alanine into the same fraction in a parallel experiment. To relate the amount of analogue incorporated into the exopenicillinase-type protein with the change in specific enzyme activity (enzyme units/μg. of exopenicillinase-type protein), the following experiment was carried out. A culture of *B. cereus* 569/H growing exponentially in 18AA medium was centrifuged, the organisms were washed in 16AA(−Phe−Val) medium and divided into three equal batches and resuspended to a density of 0·1 mg. dry wt. of bacteria/ml. under the following conditions. Batch 1 was resuspended in 16AA(−Phe−Val) medium containing 20 μg. of L-[^{14}C]valine. Samples taken from this culture at intervals were used to prepare normal [^{14}C]valine-labelled exopenicillinase. The amount of radioactivity incorporated into these samples, together with their enzymic activity, showed the specific valine content of normal exopenicillinase to be about 4·6 %, which is in good agreement with previous values (see Table 3). Batch 2 was resuspended in 16AA(−Val−Phe) medium containing L-[^{14}C]valine and unlabelled fluorophenylalanine. Samples were taken from this culture at the same intervals as culture 1 and treated to give the purified exopenicillinase fraction. The valine content of this preparation (by virtue of the arguments set out above) gave an estimate of the quantity of exopenicillinase-type protein, (column 4; Table 5, part i). Comparison of the enzyme activity (column 2; Table 5, part i) with the protein content of the purified samples (column 4) gave a measure of the specific enzyme activity (column 5). This was found to be normal at the beginning of the experiment but fell as incubation in the presence of the analogue continued and reached a value about 45 % of normal (column 6). Batch 3 was incubated in 16AA(−Val−Phe) medium containing unlabelled L-valine and *p*-fluorophenyl-[β-^{14}C]alanine. Samples were taken for this culture and treated exactly as with culture (2). In this case the radioactivity content is a measure of *p*-fluorophenylalanine incorporation and, since the growth curves for cultures 2 and 3 are almost identical, it is possible to relate the incorporation of the analogue to the [^{14}C]valine content of culture 2 and thus to the appearance of exopenicillinase-type protein and enzyme activity. The results of this part of the experiment are shown in Table 5, part ii. At the beginning of the experiment, when no effect of the analogue on specific enzyme activity could be detected in culture 2 (column 6), no *p*-fluorophenylalanine could be detected in the exopenicillinase-type protein of culture 3 (columns 9 and 11). As incubation in the presence of the analogue continued and the specific enzyme activity of the exopenicillinase-type protein fell, the incorporation of *p*-fluorophenylalanine increased. It is possible from this experiment to deduce that the exopenicillinase formed after incubation for 2½ hr. in the presence of *p*-fluorophenylalanine had 46 % of normal specific enzyme activity (Table 5, part i; column 6) and a *p*-fluorophenylalanine content of about 3 %, by wt. (see Table 5, part ii; column 11). Assuming that the molecular weight of the exopenicillinase is not

Table 5. *Comparison of valine and* p-*fluorophenylalanine content of the exopenicillinase-type protein synthesized by* Bacillus cereus 569/H *in the presence of* p-*fluorophenylalanine*

(i) (Culture 2): incorporation of [^{14}C]valine into exopenicillinase-type protein from organisms growing in 16AA(– Phe – Val) medium + 25 μg. of unlabelled *p*-fluorophenylalanine/ml. + 20 μg. of [^{14}C]valine/ml. (0·23 μc/μmole). Values in parentheses refer to non-radioactive carrier penicillinase added to control experiments carried out with the uninduced inducible strain (569) (see Materials and Methods section); e.u., enzyme units. For other details, see text.

	Culture density (mg./ml.)	Total crude penicillinase (e.u.) (1)	Total purified penicillinase (e.u.) (2)	Recovery (activity) (%)	Total valine content of purified exopenicillinase-type protein (μg.) (3)	Total amount of purified exopenicillinase-type protein (μg.)* (4)	Specific enzyme activity (e.u./μg. of exopenicillinase protein) (5)	Percentage of normal specific activity (6)
Calc. as	... —	—	—	$\frac{(2) \times 100}{(1)}$	—	—	(2)/(4)	—
	0·13	—	—	—	—	—	—	—
	0·20	8 860	1 680	19	0·26	5·4	310	100
	0·28	11 000	3 070	28	0·49	10·2	302	97
	0·42	35 600	8 200	32	2·02	43·0	192	61
	0·47	28 300	11 600	41	3·22	68·5	168	54
	0·58	33 700	12 200	36	4·04	86·0	143	46
Control	0·61	(44 000)	(16 700)	38	0·25	—	—	—

* Assuming a value for valine content of purified exopenicillinase at 4·6% (see above and Table 3).

(ii) (Culture 3): incorporation of *p*-fluorophenyl[β-^{14}C]alanine into exopenicillinase-type protein from organisms growing in 16AA(– Phe – Val) medium + 25 μg. of *p*-fluorophenyl[β-^{14}C]alanine/ml. (0·55 μc/μmole) + 20 μg. of unlabelled L-valine/ml. Values in parentheses refer to non-radioactive penicillinase added to control experiments carried out with the uninduced inducible strain (569); e.u., enzyme units. For other details, see text.

	Culture density (mg./ml.)	Total crude penicillinase (e.u.) (7)	Total purified penicillinase (e.u.) (8)	Recovery (activity) (%)	Total *p*-fluorophenylalanine content of purified exopenicillinase-type protein (μg.) (9)	True total amount of exopenicillinase-type protein (μg.)† (10)	*p*-Fluorophenylalanine content of exopenicillinase-type protein (%) (11)	Net no. of residues of *p*-fluorophenylalanine in exopenicillinase-type molecules (12)	Possible percentage replacement of phenylalanine residues‡
Calc. as	... —	—	—	$\frac{(8) \times 100}{(7)}$	—	$\frac{(4) \times (8)}{(2)}$	$\frac{(9) \times 100}{(10)}$	—	—
	0·13	—	—	—	—	—	—	—	—
	0·20	8 750	2 350	27	0	7·6	0	0	Nil
	0·285	11 100	2 330	21	0·11	7·7	1·4	1·9	28
	0·42	24 500	6 860	28	0·86	35·8	2·4	4·3	61·5
	0·47	27 400	8 500	31	1·56	50·2	3·1	5·4	77
	0·56	32 000	12 500	39	2·8	87·5	3·2	5·6	80
Control	0·61	(44 000)	(14 700)	33	0	(47·0)	0	0	0

† Valine content of exopenicillinase-type protein at a given moment is assumed to be the same as that for culture 2: see (i) above.
‡ Assuming seven phenylalanine residues/mole of exopenicillinase (S. Jacobs, unpublished work).

affected by the incorporation of *p*-fluorophenylalanine, these results correspond to a net incorporation of between five and six *p*-fluorophenylalanine residues/molecule of exopenicillinase-type protein (Table 5, part ii; column 12).

It was not possible to know from these experiments whether the *p*-fluorophenylalanine was incorporated into the exopenicillinase-type protein at the expense of phenylalanine, nor was it possible to investigate this problem by studying the effect of *p*-fluorophenylalanine on the incorporation of [^{14}C]phenylalanine into the exopenicillinase-type protein in experiments analogous to those involving [^{14}C]valine. It was found that exogenous [^{14}C]-phenylalanine was used preferentially by the organisms and even an external ratio of 20 parts of *p*-fluorophenylalanine to 1 part of phenylalanine led to an uptake of phenylalanine in preference to the *p*-fluoro derivative. Further, it has not proved possible to use any specific precursor of phenylalanine which does not interfere with *p*-fluorophenylalanine assimilation. The only radioactive precursor available was [^{14}C]glucose, which was converted into phenylalanine in about 0·1 % yield and in addition labelled most of the amino acids of the protein fraction to an appreciable extent, even in 17AA(−Phe) medium.

In these circumstances therefore it was decided to study the effect of *p*-fluorophenylalanine on the uptake of phenylalanine into various protein fractions that could be isolated readily from cultures of *B. cereus* 569/H, as this should give an indirect measure of phenylalanine replacement in the exopenicillinase-type protein.

Replacement of phenylalanine in total cell protein and total exocellular protein by p-*fluorophenylalanine*

The effect of *p*-fluorophenylalanine on the phenylalanine content of total cell protein was studied by following the appearance of phenylalanine, tyrosine and *p*-fluorophenylalanine in the protein + cell-wall fraction of organisms incubated in the presence of the analogue. The phenylalanine and tyrosine may each be estimated directly on the chromatograms but *p*-fluorophenylalanine does not separate from leucine + isoleucine in the solvent used in these experiments and must be estimated indirectly. This was done by carrying out the initial incubation in the presence of *p*-fluorophenyl-[β-^{14}C]alanine. Estimation of the ^{14}C content of the combined leucine + isoleucine and *p*-fluorophenylalanine spot then gives a measure of the amount of analogue present. For this experiment, organisms growing exponentially in 17AA(−Phe) medium were centrifuged, washed in similar medium and resuspended to a density of 0·1 mg. dry wt. of bacteria/ml. in 17AA(−Phe) medium containing *p*-fluorophenyl[β-^{14}C]alanine. A culture growing in 18AA medium provided a control. These cultures were incubated under standard conditions and samples taken at intervals were treated to yield the protein + cell-wall fraction, which was then hydrolysed, as described in the Materials and Methods section, to yield free amino acids. The hydrolysates were transferred quantitatively to chromatograms and separated to isolate phenylalanine, tyrosine and leucine + isoleucine (+*p*-fluorophenylalanine, if present). The phenylalanine and tyrosine were estimated quantitatively and the *p*-fluorophenylalanine by the ^{14}C content of the combined leucine + isoleucine and *p*-fluorophenylalanine spot (see Materials and Methods section). Fig. 4 shows the

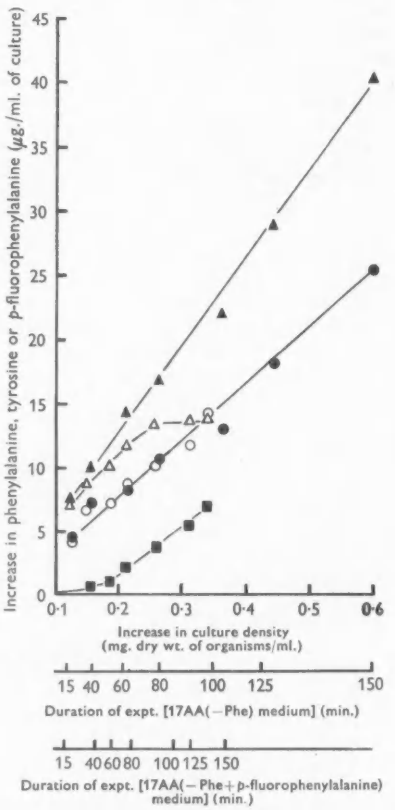

Fig. 4. Effect of *p*-fluorophenylalanine on appearance of amino acids in total cell protein. 17AA(−Phe) medium: ▲, phenylalanine; ●, tyrosine. 17AA(−Phe + *p*-fluorophenylalanine) medium: △, phenylalanine; ○, tyrosine; ■, *p*-fluorophenylalanine. L-[^{14}C]Valine, 0·23 μc/μmole; *p*-fluorophenyl[β-^{14}C]alanine, 0·55 μc/μmole.

Table 6. *Replacement by* p-*fluorophenylalanine of phenylalanine residues in total cell protein formed by cultures of* Bacillus cereus 569/H *growing in the presence of* p-*fluorophenylalanine*

Organisms growing exponentially in 17AA(– Phe) medium were harvested by centrifuging and resuspended in 17AA(– Phe) medium or 17AA(– Phe) medium containing 50 μg. of *p*-fluorophenyl[β-^{14}C]alanine/ml. (0·55 μc/μmole). Samples of bacteria taken at intervals were treated to yield the protein + cell-wall fraction. The phenylalanine, tyrosine and *p*-fluorophenylalanine content of the cell protein was estimated as described in the text.

17AA(– Phe) medium

	Culture density (mg./ml.)	Tyrosine content of protein + cell wall (μg./mg. dry wt. of organisms) (1)	Phenylalanine content of protein + cell wall (μg./mg. dry wt. of organisms) (2)	Phenylalanine/tyrosine ratio (3)
Calc. as ...	—	—	—	(2)/(1)
Sample no.				
1	0·12	41	68	1·68
2	0·215	39	68	1·75
3	0·265	40	63	1·59
4	0·35	38	62	1·61
5	0·43	40	61	1·64
6	0·60	42	60	1·64

17AA(– Phe) medium + *p*-fluorophenylalanine

	Culture density (mg./ml.)	Tyrosine content of protein + cell wall (μg./mg. dry wt. of organisms) (4)	Observed phenylalanine content of protein + cell wall (μg./mg. dry wt. of organisms) (5)	Theoretical phenylalanine content of protein + cell wall (μg./mg. dry wt. of organisms) (6)
Calc. as ...	—	—	—	(4) × (3)
Sample no.				
1	0·12	38	63	64
2	0·185	41	59	66
3	0·22	39	52	65
4	0·265	41	48	66
5	0·29	38	44	64
6	0·34	41	38	65

17AA(– Phe) medium + *p*-fluorophenylalanine (cont.)

	Culture density (mg./ml.)	Difference between theoretical and observed phenylalanine content of protein + cell wall (μg./mg. dry wt. of organisms) (7)	*p*-Fluorophenyl[β-^{14}C]-alanine content of protein + cell wall (μg./mg. dry wt. of organisms) (8)	Replacement of phenylalanine by *p*-fluorophenylalanine (%) (9)
Calc. as ...	—	(6)–(5)	—	$\frac{(8) \times 100}{(7)}$
Sample no.				
1	0·12	0	0	—
2	0·185	7	4·3	61
3	0·22	13	8·6	66
4	0·265	18	12	66
5	0·29	20	17·2	86
6	0·34	27	21	78

differential effect of *p*-fluorophenylalanine on the appearance of these amino acids in the protein + cell-wall fraction. The analogue has no effect upon the differential rate of incorporation of tyrosine. The incorporation of phenylalanine, however, is markedly depressed. After incubation for 60 min. in the presence of the analogue, the differential rate of incorporation is about 40 % of normal, and after incubation for 2 hr. the flow of phenylalanine to protein has practically ceased. On the other hand, no *p*-fluorophenylalanine is detected in the cell protein at the beginning of the experiment. Subsequently, however, the differential rate of *p*-fluorophenylalanine incorporation increases for about 2 hr., after which the rate remains constant. To calculate the degree of 'replacement' of phenyl-

alanine by *p*-fluorophenylalanine, it is necessary to know how much phenylalanine would have been incorporated if the *p*-fluoro derivative had no differential effect upon the incorporation of natural amino acid. This may be deduced from the tyrosine content of the protein under these conditions (see Table 6). As shown in Fig. 4, *p*-fluorophenylalanine has no effect on tyrosine uptake into protein. As the phenylalanine–tyrosine ratio of normal protein is about 1·6:1 (see Table 6, columns 1–3) it is possible to calculate the theoretical phenylalanine content of protein synthesized in the presence of the analogue (Table 6, column 6). For complete 'replacement' of phenylalanine by the *p*-fluoro derivative, the difference between the theoretical and observed phenylalanine contents of protein (Table 6, columns 5–7) would have to be the same as the *p*-fluorophenylalanine content observed at the same time (Table 6, column 8). In fact the *p*-fluorophenylalanine content accounts for about 65–85 % of the displaced phenylalanine. This could amount to complete replacement within the error of the method.

This experiment shows therefore that after growth for about 2 hr. in the presence of *p*-fluorophenylalanine, the flow of phenylalanine to protein is almost completely suppressed in favour of the *p*-fluoro derivative. In separate experiments, similar effects were obtained with *p*-fluorophenylalanine on the total exocellular-protein fraction and therefore under these conditions a high degree of replacement of phenylalanine in exopenicillinase-type protein by *p*-fluorophenylalanine might be expected, particularly in the later stages of inhibition.

Immunological properties of exopenicillinase in relation to incorporation of p-*fluorophenylalanine*

Experiments (Richmond, 1960) have shown that exopenicillinase synthesized in the presence of *p*-fluorophenylalanine has abnormal neutralization characteristics when titrated with anti-exopenicillinase γ-globulin. As the abnormality of the enzyme increased during the initial incubation for 2 hr. in the presence of the analogue, it seemed possible that this was related to the increasing content of *p*-fluorophenylalanine and the concomitant fall in specific enzyme activity (enzyme units/μg. of exopenicillinase-type protein).

To relate these processes more closely, the previous experiment was repeated and samples of exopenicillinase were tested for their 'abnormality' in the neutralization reaction. Table 7 compares the level of incorporation of analogue with the specific enzyme activity and the immunological properties of the enzyme, measured in terms of the plateau zone, the neutralization slope and the residual enzyme activity. The analogue content of the exopenicillinase rises during the initial incubation for

Table 7. *Relationship between incorporation of* p-*fluorophenylalanine and immunological properties of exopenicillinase synthesized in the presence of the analogue*

Bacteria growing exponentially in 17AA(– Phe) medium were centrifuged and the organisms resuspended in 17AA(– Phe) medium containing 50 μg. of *p*-fluorophenyl[β-^{14}C]alanine/ml. (0·55 μc/μmole). Duplicate samples taken at intervals were treated: (*a*) to determine the *p*-fluorophenylalanine content of the exopenicillinase-type protein; (*b*) to determine the neutralization characteristics of the exopenicillinase-type protein synthesized in the presence of the analogue. The neutralization characteristics of a normal exopenicillinase preparation are included for comparison; e.u., exopenicillinase units; n.u., neutralization units.

Interval after addition of the analogue (min.)	Culture density (mg./ml.)	*p*-Fluorophenyl-[β-^{14}C]alanine content of purified exopenicillinase (%, by wt.)	Possible degree of replacement of phenylalanine by *p*-fluorophenylalanine (%)	Specific enzyme activity (e.u./μg. of enzyme protein)	Percentage of normal specific enzyme activity	Immunological properties*		
						Plateau zone (n.u.)	Neutralization slope (n.u./0·1 ml.)	Residual activity (%)
0	0·13	—	—	—	—	—	—	—
45	0·20	0	0	307	98	8 ± 1	35	5·8
60	0·275	1·7	43	245	78	12 ± 1	26	5·8
80	0·34	1·9	48	220	70	13 ± 1	23	8·8
100	0·39	2·8	71	165	53	18 ± 2	15	8·7
120	0·46	3·0	76	150	48	25 ± 3	14	14·2
180	0·68	2·7	68	155	49	27 ± 3	14	12·0
Normal enzyme	—	Nil	Nil	312	100	7 ± 1	36	5·4

* Tested at 30 e.u./ml

2 hr. and this is accompanied by a fall in specific enzyme activity and increased immunological abnormality. Subsequently the steady differential rate of *p*-fluorophenylalanine incorporation is associated with approximately constant enzymic and immunological properties. As discussed by Richmond (1960), the abnormal properties of the exopenicillinase synthesized under these conditions could be partly explained on a basis of lowered specific enzyme activity. In fact, the fall in specific activity found in this experiment (50–60%: cf. Table 6, part i) is about sufficient to account for the abnormal neutralization slope exhibited in the neutralization test.

DISCUSSION

The overall pattern of changes after the addition of *p*-fluorophenylalanine to cultures of *B. cereus* are similar to those reported for *E. coli* (Munier & Cohen, 1956, 1959) and yeast (Cohen, Halvorson & Spiegelman, 1959). There are, however, certain differences, the effects of the analogue being neither so immediate nor so clear-cut. Growth of *B. cereus* is markedly inhibited by the analogue, but linear growth does not commence immediately (Richmond, 1960). For about 45 min. after the addition of the analogue to cultures growing exponentially, the growth rate is slowed but still greater than linear. Subsequently a period of 'linear' growth occurs, during which the rate of increase in dry weight of the culture slowly falls over a period of 3–4 hr. If the analogue were causing inhibition of the division process but the mass of the culture were increasing at a linear rate, enlarged forms of the organisms would be expected. In fact, the mean length of organisms was only slightly increased even after 8 hr. in the presence of the analogue (M. H. Richmond & J. Collins, unpublished work) and therefore division of *B. cereus* must occur in the presence of *p*-fluorophenylalanine (cf. Cohen *et al.* 1959). The differential rate of *p*-fluorophenyl[β-^{14}C]alanine incorporation into the protein fraction of *B. cereus* increases over the first 45 min. of incubation and then becomes constant. This time course of incorporation is distinctly different from that obtained in *E. coli* (Munier & Cohen, 1956, 1959), where the differential rate of incorporation is constant from the moment of addition of the analogue to the system. It is possible that the delayed effect of the analogue in *B. cereus* is due to a high internal 'pool' concentration of phenylalanine. This amino acid is known to compete effectively with *p*-fluoro derivative when the amino acids are present together in the growth medium. However, the 'pool' concentration of phenylalanine in *B. cereus* is relatively low (approx. 0·8 μmole/g. dry wt. of organisms; cf. glutamic acid at 8 μmoles/g.) and this quantity of phenylalanine would suppress *p*-fluorophenylalanine incorporation into protein for only about 15 min. at the observed growth rates.

A more likely explanation of the delayed effect of the analogue lies in the effect of this compound on the biosynthetic pathway of phenylalanine. It is known (Richmond, 1960) that phenylalanine is an efficient competitor for the *p*-fluoro derivative in the assimilation system when the two amino acids are present together in the growth medium. It seems possible therefore that, once inside the cell, the analogue may inhibit the endogenous synthesis of phenylalanine and only after this has occurred may the analogue act fully in the protein-biosynthetic system. Inhibition of the function of biosynthetic pathways has been reported for a number of amino acid analogues [e.g. 4-methyltryptophan inhibition of tryptophan synthetase, Trudinger & Cohen (1956)] and Munier & Cohen (1959) tentatively suggest that *p*-fluorophenylalanine inhibits the biosynthesis of phenylalanine in *E. coli*. Certainly *p*-fluorophenylalanine does not inhibit the biosynthesis of phenylalanine in *B. cereus* immediately since it can be shown that phenylalanine continues to be synthesized for more than 10 min. after addition of the analogue and is then incorporated into cell protein (M. H. Richmond, unpublished work). A delayed inhibition by *p*-fluorophenylalanine of the biosynthetic supply of phenylalanine could therefore be responsible for the delayed effect of the analogue on the protein-synthesizing system.

In the experiments reported above, the rate of incorporation of *p*-fluorophenylalanine into all the protein fractions studied increased and reached a maximum value after incubation for 1–2 hr. In this way the incorporation of the analogue into purified exopenicillinase increased to a maximum rate, at which the incorporation of *p*-fluorophenylalanine was equivalent to the majority of the seven available phenylalanine residues. Although it has not been proved that the incorporation of the analogue into exopenicillinase is at the expense of the natural amino acid, indirect evidence points strongly to this conclusion. The analogue does not seem to have a specific inhibitory effect on any single fraction of protein (see also Cowie, Cohen, Bolton & de Robichon-Szulmajster, 1959). Replacement of a large proportion of the phenylalanine by the *p*-fluoro derivative can be shown to occur both with the total cell-protein fraction and also with the total exocellular protein. If the same is not true of the exopenicillinase, it is necessary to invoke a specific effect of the analogue on this protein, a possibility for which there is no experimental support.

The previous paper (Richmond, 1960) has described the immunological properties of exo-

penicillinase synthesized in the presence of *p*-fluorophenylalanine. An attempt has been made therefore to explain the immunological findings on the basis of the abnormal chemical properties of the exopenicillinase-type protein.

As crystalline exopenicillinase from this strain of *B. cereus* contains seven phenylalanine residues (S. Jacobs, personal communication), and as the majority can be replaced by the analogue after incubation for 2 hr., it is important to know whether this represents a random replacement of residues or specific replacement of the same residues. The experiments reported in this paper favour random replacement. However, even if incubation in the presence of the analogue is continued for long periods it has not proved possible to show complete 'replacement'. The situation is complicated by the fact that the exopenicillinase fraction normally contains molecules, made soon after the addition of the analogue, which are known to have a low degree of replacement and this means that complete replacement can never be attained. However, after 4 hr. in the presence of the analogue, the degree of replacement should be greater than a net value of six residues. It is possible, however, that complete 'replacement' does occur for the following reasons. First the methods used to purify the abnormal enzymes would not detect an 'exopenicillinase' protein which had no enzyme activity and which did not react with the specific anti-exopenicillinase antiserum. Complete replacement could lead to such a protein. Secondly, the method used for measuring the degree of replacement is subject to very large errors and a value of five to six could easily represent complete replacement. Certainly, very little phenylalanine seems to appear in the total cell-protein fraction after 2 hr. in the presence of *p*-fluorophenylalanine, whereas the analogue continues to be incorporated (see Table 6).

It is certain that the addition of *p*-fluorophenylalanine to cultures of *B. cereus* leads to the formation of at least one type of abnormal exopenicillinase molecule with impaired specific enzyme activity.

If five to six residues of phenylalanine are randomly replaced this would lead to the formation of about 50 different types of 'penicillinase' molecule and it is not possible to predict anything about the enzymic or immunological properties of these proteins. Experimentally, it can be shown (Richmond, 1960) that the net immunological reaction of the exopenicillinase-type protein is impaired at low antibody–enzyme ratios; the neutralization slope with normal antiserum is lowered and the residual enzyme activity of the preparation, on addition of three equivalent doses of antiserum, is slightly higher than normal. As has been shown above (see Table 7), the low neutralization slope and high residual activity can be accounted for on the basis of the low net specific enzyme activity of the abnormal exopenicillinase preparation. It is not possible, however, to account for the broad 'plateau zone' found on neutralizing abnormal enzyme preparations. Normal enzyme shows a slight plateau and some of the increase found with the abnormal enzyme could be due to a lowered affinity of some or all the components of the mixture for the antiserum. As discussed by Richmond (1960), neutralization of a complex mixture of related but differing proteins with a complex mixture of antibody molecules is difficult to analyse.

These findings suggest that it might be possible to separate a number of 'abnormal' exopenicillinase molecules by physicochemical methods. However, so far the abnormal mixture has been found to behave identically with the normal enzyme in immune electrophoresis by the method of Grabar (1959) and Grabar & Williams (1955), and also on attempted separation in the gradient-electrophoresis apparatus (Charlwood & Gordon, 1958). This may reflect the similar physicochemical properties of phenylalanine and its *p*-fluoro derivative when combined in proteins.

SUMMARY

1. Cultures of *Bacillus cereus* 569/H incubated in fully synthetic amino acid medium lacking phenylalanine but containing *p*-fluorophenylalanine incorporate the unnatural amino acid into total cell protein, total exocellular protein and exopenicillinase of the organisms.

2. The incorporation of *p*-fluorophenylalanine into the total cell-protein and total exocellular-protein fractions of the organisms is associated with a high degree of replacement of the phenylalanine normally appearing in these fractions by the *p*-fluoro derivative. The degree of replacement on a differential basis increases for about 90 min. before reaching a maximum rate.

3. Incorporation of *p*-fluorophenylalanine into the exopenicillinase prepared from the organisms leads to the formation of at least one species of molecule with impaired specific enzyme activity and abnormal neutralization characteristics.

4. The relationship between the abnormal chemical and immunological properties of the exopenicillinase-type protein formed in the presence of *p*-fluorophenylalanine is discussed.

I would like to express my indebtedness to Dr M. R. Pollock for providing the anti-penicillinase sera and for much stimulating discussion in connexion with the work. I also wish to thank Mr Norman Dickens and Mr Rex Pyke for valuable technical assistance.

REFERENCES

Boyd, W. C. (1956). *Fundamentals of Immunology*, 2nd ed. New York: Interscience Publishers Inc.
Charlwood, P. A. & Gordon, A. H. (1958). *Biochem. J.* **70**, 433.
Cohen, G. N., Halvorson, H. O. & Spiegelman, S. (1959). In *Microsomal Particles and Protein Synthesis*, p. 100. Ed. by Roberts, R. B. New York: Pergamon Press Ltd.
Cohen, G. N. & Munier, R. (1959). *Biochim. biophys. Acta*, **31**, 347.
Cowie, D., Cohen, G. N., Bolton, E. T. & de Robichon-Szulmajster, H. (1959). *Biochim. biophys. Acta*, **34**, 39.
Grabar, P. (1959). *Meth. biochem. Anal.* **7**, 1.
Grabar, P. & Williams, C. A., jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
Halvorson, H. O. & Spiegelman, S. (1952). *J. Bact.* **64**, 207.
Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
Kruh, J. & Rosa, J. (1959). *Biochim. biophys. Acta*, **34**, 561.
Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. S. (1951). *J. biol. Chem.* **193**, 265.
McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Mitchell, P. D. (1949). *J. gen. Microbiol.* **4**, 399.
Munier, R. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
Munier, R. & Cohen, G. N. (1959). *Biochim. biophys. Acta*, **31**, 378.
Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
Richmond, M. H. (1959). *Biochem. J.* **73**, 155.
Richmond, M. H. (1960). *Biochem. J.* **77**, 112.
Trudinger, P. A. & Cohen, G. N. (1956). *Biochem. J.* **62**, 488.
Urbá, R. C. (1959). *Biochem. J.* **71**, 513.
Vaughan, M. & Steinberg, D. (1958). *Fed. Proc.* **17**, 328.

Biochem. J. (1960) **77**, 135

Argininosuccinic Aciduria: Identification and Reactions of the Abnormal Metabolite in a Newly Described Form of Mental Disease, with Some Preliminary Metabolic Studies

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Allan, Cusworth, Dent & Wilson (1958) have reported a new form of severe mental disease which they believe to be due to an inborn error of metabolism. The authors described a family with four children in which two of the sibs were normal and the other two were seriously mentally retarded. Paper-chromatographic examination of the urine of the family showed that the two affected children were excreting large amounts of a ninhydrin-reacting substance which was absent in the urine of the rest of the family. Moreover, further tests on the affected children showed a relatively high concentration of the abnormal metabolite in the cerebrospinal fluid and a somewhat lower concentration in the plasma. The unknown substance could not be identified as any of the substances usually found in normal urine, nor with any of the compounds found only in trace amounts (Westall, 1955). Hence at the time when the full report of the case was published the particular metabolite was unidentified but the authors were of the opinion that it was probably a peptide of a type resistant to acid hydrolysis.

Further work on this substance (reported briefly by Westall, 1958) has now established that it is argininosuccinic acid. This acid was first postulated on theoretical grounds to be an intermediate in the pathway of synthesis of arginine from citrulline and aspartic acid in the presence of liver enzymes (Ratner & Pappas, 1949). It was later synthesized, isolated and characterized from a similar system (Ratner, Petrack & Rochovansky, 1953). These authors demonstrated that the reaction takes place in two stages. In the first, argininosuccinic acid is formed from citrulline and aspartic acid under the action of a 'condensing enzyme' present in mammalian liver and kidney; this requires adenosine triphosphate. The second stage requires the presence of a 'splitting enzyme', which catalyses the breakdown of argininosuccinic acid to form arginine and fumaric acid. They were able to prepare argininosuccinic acid from both of these systems since the splitting-enzyme system is reversible. The natural accumulation of argininosuccinic acid in mammalian tissues or body fluids has not been demonstrated before and it may be

assumed that under normal conditions it occurs only transiently. However, it has been found in pea-meal extracts (Davison & Elliott, 1952) and a mutant strain of *Neurospora crassa* has been described which accumulates argininosuccinic acid in the mycelium (Fincham & Boylen, 1955).

In the present paper details are given of the formal identification of argininosuccinic acid obtained for the first time from human material.

RESULTS

Chemical studies

Isolation of argininosuccinic acid from the patient's urine. Several attempts were made to isolate the unknown substance by using a system of coupled ion-exchange-resin columns (Westall, 1952), but the results were extremely confusing and it became obvious that the substance being studied was being degraded during fractionation. However, it was possible to isolate a white crystalline substance which moved to a different position on paper chromatography from that taken up by the original unknown substance. This newly isolated substance was stable to acid hydrolysis but gave aspartic acid and ornithine after alkaline hydrolysis. This information provided the clue and led to the recognition that the original substance was probably argininosuccinic acid (ASA) and that the isolated crystalline substance was one of the cyclic forms described by Ratner *et al.* (1953).

As soon as it was supposed that the unknown substance was ASA a simple method for its isolation was immediately available. Ratner *et al.* (1953), in their studies on the biosynthesis of ASA, successfully isolated it from tissue homogenates as the barium salt, which is soluble in water but virtually insoluble in aqueous 75% ethanol. This method works equally well for isolation of ASA from urine, and 160 g. of the crude barium salt was obtained from 25 l. of urine from one of the affected sibs (K.R.). As Ratner *et al.* (1953) pointed out, ASA does not in practice take up the expected 3 equiv. of barium but has a variable content of between 2 and 2·5 equiv. of barium when precipitated in the presence of excess of $Ba(OH)_2$ at about pH 10–11. ASA is conveniently stored as the barium salt, which seems to be stable if kept dry. The production of the free base presents some difficulty since it forms cyclic compounds spontaneously in aqueous solution. The anhydride formation is favoured by high temperatures and acidic conditions. Hence if an aqueous solution of ASA remains at room temperature for a short time considerable cyclization takes place, since the isoelectric point of the free ampholyte is about pH 3·4. The time required to remove barium quantitatively as $BaSO_4$ and to produce a freeze-dried powder leads to the formation of about 10% of anhydride. However, removal of barium by exchange with NH^+_4 ion on a cation-exchange-resin column at 2° and freeze-drying of the effluent produced a much purer sample of ASA. This method of producing the free base is an apparent contradiction of the statement made earlier that ASA is unstable on ion-exchange resins. This can be explained by the fact that the conditions were carefully selected to avoid cyclization. The temperature was kept at 2°, the amount of resin used was only slightly more than that required to remove barium and exchange with NH_4^+ ion. Thus ASA did not exist as the free acid until NH_3 was removed by volatilization during the freeze-drying. Subsequent analysis gave no evidence for ammonium salt formation. The passage of the solution through the resin column was completed in 4–5 min. Unfortunately, in spite of the limited anhydride formation it has still not been possible to secure crystalline ASA. Therefore the example of Ratner *et al.* (1953) has been followed and the anhydride forms, which crystallize readily from aqueous ethanol, have been used for elementary analyses.

Behaviour of argininosuccinic acid on filter-paper chromatograms. Fig. 1 (*a*) represents the standard two-way paper chromatogram most commonly used for screening urines to detect amino acidurias of various types (Dent, 1951). Aspartic acid, glutamic acid and glycine were put in as markers since the lutidine–water solvent runs off the leading edge of the paper and absolute R_F values do not apply. When the purified barium salt of ASA, obtained by repeated precipitation of the crude material, was run in this system a single spot was obtained in the position indicated. A sample of Dr Ratner's barium salt of ASA moved to the same position. When, however, free ASA, regenerated from the barium salt, was dissolved in water and allowed to stand at room temperature before being examined by paper chromatography three well-defined spots were obtained. One corresponded with the original position of ASA and the others took up the positions marked as B and C. Ratner *et al.* (1953) were aware that ASA changed into a modified form, on standing in aqueous solution, which they postulated to be a cyclic anhydride. They isolated and analysed this substance. When a sample of this anhydride (this and the sample of barium argininosuccinate were kindly supplied by Dr J. B. Jepson from gift samples from Dr S. Ratner) was run in the same chromatographic system it moved to the position marked C. Thus it would appear that there are two modified, probably both cyclic, forms of ASA. It is understandable that Ratner *et al.* (1953) were not aware of this because they used one-way paper chromatograms

with phenol–NH_3 as the solvent and in this system the R_F values of ASA and the B substance differ only slightly.

Behaviour of argininosuccinic acid and its anhydrides on high-voltage paper electrophoresis diagrams. As has been shown above, ASA and its two 'cyclic' forms B and C can be separated on two-way paper chromatograms, but a quicker and more convincing separation can be achieved by high-voltage paper electrophoresis. The apparatus used was a slightly modified form of that described by Gross (1955). A Whatman 3MM paper strip 10 cm. wide and 52 cm. long was dipped in buffer and blotted with moderate pressure. The samples to be analysed were spotted on the starting line, which was at various positions, according to the expected direction of migration of the substances. The paper strip was then placed between water-cooled metal plates 45 cm. long, but protected from contact with the plates by sheets of polythene 0·005 in. thick. An even pressure of 5 lb./in.^{2} was then applied to the bottom plate by means of an inflated rubber bag. The upper plate, after assembly, was not free to move. The ends of the paper strip were then connected by means of wet filter-paper wicks to polythene buffer pots in which the electrodes from the power pack were immersed. For the separation of ASA and its anhydrides a pyridine–acetate buffer, pH 5·1, was used (pyridine, 25 ml.; acetic acid, 10 ml., diluted to 2·5 l.). Fig. 1 (*b*) shows a typical ninhydrin-developed strip illustrating the separation of ASA, B and C after running for 20 min. with a potential difference between the ends of the strip of 6 kv and passing a current measured at 60 ma. In this buffer system ASA (isoelectric point 3·4) has an appreciable net negative change and moves towards the anode; B (isoelectric point 4·2) also has a slight net negative charge and moves less rapidly to the anode; C (isoelectric point 5·5) moves slightly towards the cathode.

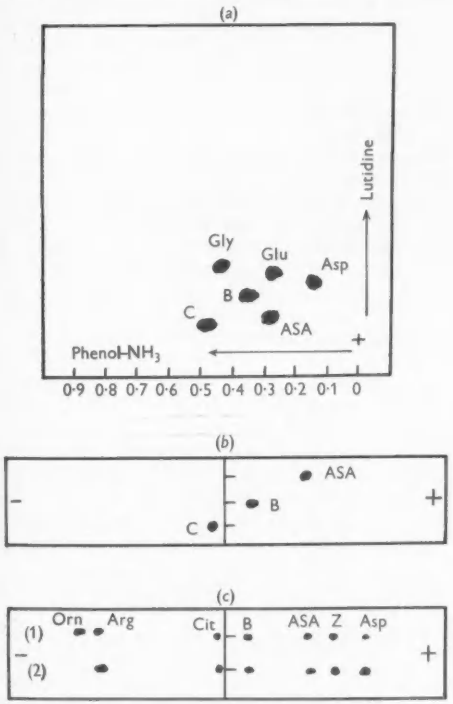


Fig. 1. (*a*) A two-way paper chromatogram with added marker substances showing the positions taken up by ASA and the two anhydrides B and C. (*b*) A paper-electrophoresis diagram showing the relative positions of ASA, B and C after running for 20 min. in pyridine–acetic acid buffer, pH 5·1, at 130 v/cm., 60 ma. (*c*) A paper electrophoresis diagram run as in (*b*) and showing the products obtained after hydrolysis of ASA with alkali: (1), 0·2 N-$Ba(OH)_2$ for 16 hr. at 105°; (2), 0·5 N-$Ba(OH)_2$ for 16 hr. at 105°. The samples were applied to the paper at the centre line. The positions of the substances were shown up by spraying with 0·1 % ninhydrin in butanol.

Stability of argininosuccinic acid and its two cyclic forms. In order to study the behaviour of ASA in water more closely, a series of experiments were devised to study the effect of pH and temperature on solutions of ASA, B and C. A series of buffer solutions of pH 2–11 (Theorell & Stenhagen, 1939) were made up (0·1 M) and these were mixed with equal volumes of 0·02 M-solutions of the substances being investigated. One series was allowed to stand at room temperature (22°) for 48 hr. and a second series was heated at 100° for 1 hr. The percentage composition of the equilibrium mixtures was estimated by visual comparison of the spots obtained after electrophoresis of the samples with spots obtained from standard amounts of the substances run under the same conditions and the results, which are not claimed to have an accuracy greater than ±10 %, are presented graphically in Fig. 2. When ASA, in solution, is allowed to stand at 22° [Fig. 2 (*a*)], it is relatively stable, provided that the pH is adjusted to be more alkaline than 6, but under more acidic conditions it is converted into the B and C forms, predominantly the latter. After 1 hr. at 100° [Fig. 2 (*c*)], ASA is completely transformed to about equal amounts of B and C at acid pH; at pH 5–6 the equilibrium favours the B form and in the more alkaline range some ASA survives and the cyclization is only to the B form. When B is similarly treated it is unaffected throughout the pH range after 24 hr. at 22° [Fig. 2 (*c*)], but it is slightly changed after 1 hr. at

100° [Fig. 2 (*d*)]; about 25% is changed to the C form at pH 2 and about 10% of ASA is regenerated at pH 11. The C substance is stable at pH 2–5 at 22° but is more readily converted back into ASA than B when the conditions become more alkaline [Fig. 2 (*e*)]. At 100°, C is still stable at pH 2–3 but at pH 5 the ring is opened rapidly with the formation of ASA, which presumably partly recyclizes to form B. It is curious that when C is heated at 100° at pH 5–7 there is a loss (30–40%) of total ninhydrin colour. Since the ninhydrin reaction is due to the amino group of the ornithine moiety it would seem that there may be some formation of intermediary substances involving some modification of the amino group. To summarize, ASA is more stable at alkaline pH provided that the temperature is not raised. The optimum conditions for forming the B anhydride is to heat ASA at pH 6; C, on the other hand, is best prepared by acidifying ASA and standing at room temperature.

Treatment of argininosuccinic acid and its anhydrides with strong acid. When ASA is refluxed for 24 hr. with 5·5N-HCl it is rapidly converted into the B and C forms and there is some further degradation (not exceeding 10%) leading to the formation of arginine, ornithine and aspartic acid. Similar experiments with B and C individually showed the same relative stability and the same products of degradation but there was a slight tendency for C to be more stable than B under these conditions.

Degradation studies on argininosuccinic acid with alkali. As Ratner *et al.* (1953) have observed, ASA breaks down completely to ornithine and aspartic acid after refluxing with 3N-$Ba(OH)_2$ for 24 hr. They also stated that this was true also for their anhydride (C) although the liberation of NH_3 was slower and the final yield of aspartic acid was lower than for ASA. Repeat experiments under these conditions, where the products of hydrolysis have been examined by paper electrophoresis, have confirmed that ASA and C yield ornithine and aspartic acid only. Further, B also gave the same degradation products. Presumably B and C are first converted into the open ASA forms and further degradation proceeds from there. Reference to Fig. 2 (*d*) shows that B is more resistant to ring opening at alkaline pH than C [Fig. 2 (*f*)]. In discussing the route of degradation of ASA with alkali, Ratner *et al.* (1953) considered it might be by a simple multiple cleavage to give ornithine, aspartic acid, CO_2 and NH_3, but they stated that with milder conditions there might be some citrulline formed first which would later break down to yield ornithine. With alkali treatment of their anhydride (C) they had evidence for the formation of ureido acids and postulated breakdown by a route through ornithine and 5-(acetic-acid) hydantoin, the latter being further degraded to ureidosuccinic acid and finally to aspartic acid, CO_2 and NH_3.

Degradative studies of ASA with varying strengths of alkali have shown that the series of

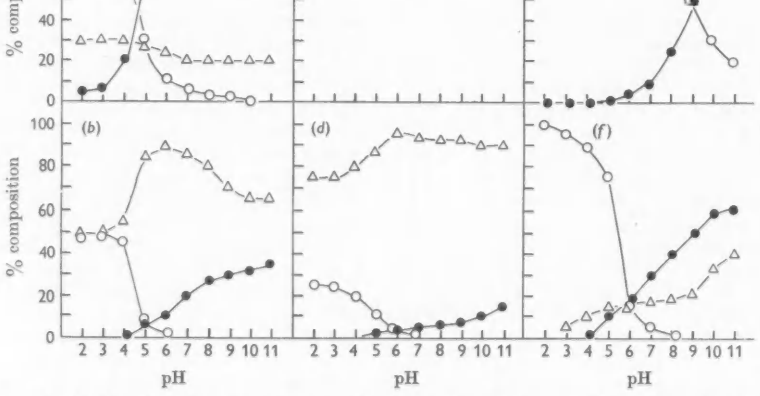


Fig. 2. Graphs showing the effect of temperature and pH on 0·01 M-solutions of ASA (●), B (△) and C (○). (*a*) ASA stood at 22° for 48 hr. (*b*) ASA heated at 100° for 1 hr. (*c*) B stood at 22° for 48 hr. (*d*) B heated at 100° for 1 hr. (*e*) C stood at 22° for 48 hr. (*f*) C heated at 100° for 1 hr.

events that leads to the ultimate production of ornithine and aspartic acid is complex. Hydrolysis with 0·2N-$Ba(OH)_2$ for 16 hr. at 105° in a sealed tube gives a number of products: arginine, ornithine, citrulline, an unknown substance 'Z' and a trace of aspartic acid together with unchanged ASA and B. When 0·5N-$Ba(OH)_2$ was used for the same hydrolysis time no arginine was found and the amount of ornithine and aspartic acid was increased. Fig. 1 (*c*) illustrates the position taken up by these substances on the high-voltage electrophoresis diagram. The B and C forms, if treated similarly, give basically the same results but no trace of the Z substance was found. The occurrence of this unknown substance was difficult to explain until it was discovered that the sample of partially purified barium argininosuccinate used in the hydrolysis studies contained, as an impurity, another unknown substance 'Y'. Y behaves as an ampholyte, since it is retained on both anion- and cation-exchange resins and can be recovered without degrading. It does not give a colour with ninhydrin nor, in fact, with any of the routine spray reagents used in the chromatographic or electrophoretic studies. It was found by chance, since its presence on a particular electrophoresis diagram caused a distortion in the shape of the spot given by a known substance in a neighbouring position on the paper strip. It has now been found that its presence on paper electrophoresis diagrams can be shown by spraying with the alkaline nitroprusside–ferricyanide reagent (Kirby-Berry, Sutton, Cain & Berry, 1951). It gives a red spot which fades within 15 min. but it can still be seen some hours later since it gives a greenish fluorescence when viewed in u.v. light. Y has now been isolated but not fully characterized. However, it is converted into Z by mild alkaline hydrolysis, which explains the occurrence of Z in the hydrolysis studies; both Y and Z yield only ornithine and aspartic acid when fully hydrolysed with 2N-NaOH for 24 hr. Hence it would seem that Y must have some close structural relationship with ASA.

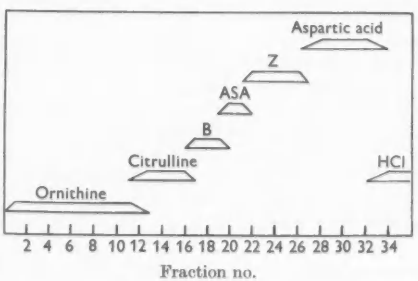


Fig. 3. Amino acid composition of the fractions obtained after displacement with 0·1N-HCl from a column of Dowex-2 ion-exchange resin. The mixture applied to the column was a partial hydrolysate of ASA with 0·5N-NaOH.

Isolation of substances yielded by mild alkali treatment of argininosuccinic acid. In order to isolate some of the degradation products of ASA with alkali, and also the contaminating unknown substance Z, an hydrolysis of 10 g. of the crude barium salt was undertaken (as described in the Experimental section). The flow diagram of separation and the amino acid composition of the fractions is shown in Fig. 3. The early fractions contain ornithine only, followed by two fractions mixed with citrulline and then pure citrulline and so on. Z was easily isolated since it has a low solubility in water and, in fact, crystallized out as a fine white powder in the fraction tubes.

Quantitative determination of argininosuccinic acid. The method of Moore & Stein (1954) for the quantitative estimation of amino acids in body fluids with a column of the cation-exchange resin Dowex-50 provides a useful means of estimating ASA. It was used by Allan *et al.* (1958) for measuring the relative concentration of their unknown metabolite (now known to be ASA) in the blood plasma, cerebrospinal fluid and urine of their patients. Their more detailed analyses are now available (Cusworth & Dent, 1960). Further work on the chemistry of ASA has now led to a probable explanation of the behaviour of ASA on such a column. In the body fluids of these patients, within the range of physiological pH values, ASA exists predominantly in the open-chain form. However, in the early stages of the passage of the samples down the Moore & Stein column, the pH of the ambient solution is at 3·4 and is even more acid (2·0) than this when the sample is applied to the column. Under these conditions ASA forms an equilibrium mixture with its two anhydrides (called for convenience B and C) in the approximate ratio of 2:3:5. Since these three substances have different isoelectric points (ASA 3·4, B 4·2, C 5·5), ASA, being more acidic, moves ahead down the column and this leads to a new equilibrium with further production of more B and C. Hence the quantity of free ASA is soon decreased to minimal amounts because, under the conditions of the column procedure, there is no regeneration of ASA from the separated anhydrides. This all takes place quite rapidly since fairly sharp twin peaks are seen on the column flow diagram, which can be shown to correspond with the cyclic forms B and C. They occur near the position taken up by leucine and isoleucine and in the urine samples from the patient under study the B and C peaks are so large that the smaller leucine and isoleucine peaks were frequently overlapped. Paper-chromatographic analysis of the fractions from the column showed

that the first of the two peaks was due to the B form and the second showed the C form with a small amount of free ASA. The occurrence of the small quantity of the open-chain form was probably an artifact and it was probably regenerated from C during the desalting of the fractions on small ion-exchange columns before the paper-chromatographic analysis. The explanation of the behaviour of ASA on the Moore & Stein column outlined above was further corroborated by carrying out test runs on the columns with isolated ASA (free base) and the B and C anhydrides. The ninhydrin colour values of B and C are 94 and 100% respectively of that given by leucine. A typical flow sheet of the Moore & Stein analysis of the urine of a child with argininosuccinic aciduria is shown in Fig. 4.

Clinical metabolic studies

Effects of dietary change on the excretion of argininosuccinic acid. Since ASA is an intermediate in the urea cycle it might be expected that variation in the quantities of the substances made available to the cycle could cause variation in the excretion of ASA.

The patient was an 8-year-old male child [previously described as case 2 of Allan *et al.* (1958)] admitted to the Metabolic Ward of University College Hospital, London, under the care of Professor C. E. Dent. The child was fed with a mixed constant diet of his own choice containing 30 g. of protein/day. The dietary supplements were fed in addition to the basic diet and were given in an orange-juice drink, one-quarter of which was consumed at each of four meals during the day. Urine samples (24 hr.) were collected throughout the study. L-Arginine was added at 2 g./day over a period of 3 days and the urine collected on the third day was selected for analysis. L-Aspartic acid, (5·0 g.) L-ornithine (4·0 g.) and L-citrulline (4·0 g.) were fed for one day only but as the last two substances are not found in the normal diet in appreciable quantities, a small test dose was given on the previous day in case of any ill-effects. The child was put back on the basic diet for several days between each experimental feed. As a final trial the protein level of the basic diet was dropped to 10 g./day over a period of 5 days, other dietary constituents being kept as constant as possible. The urine sample collected on the fifth day was selected for analysis.

The child remained physically well and active throughout the period of study and there appeared to be no appreciable change in his mental state. The results are shown in Table 1. ASA was determined

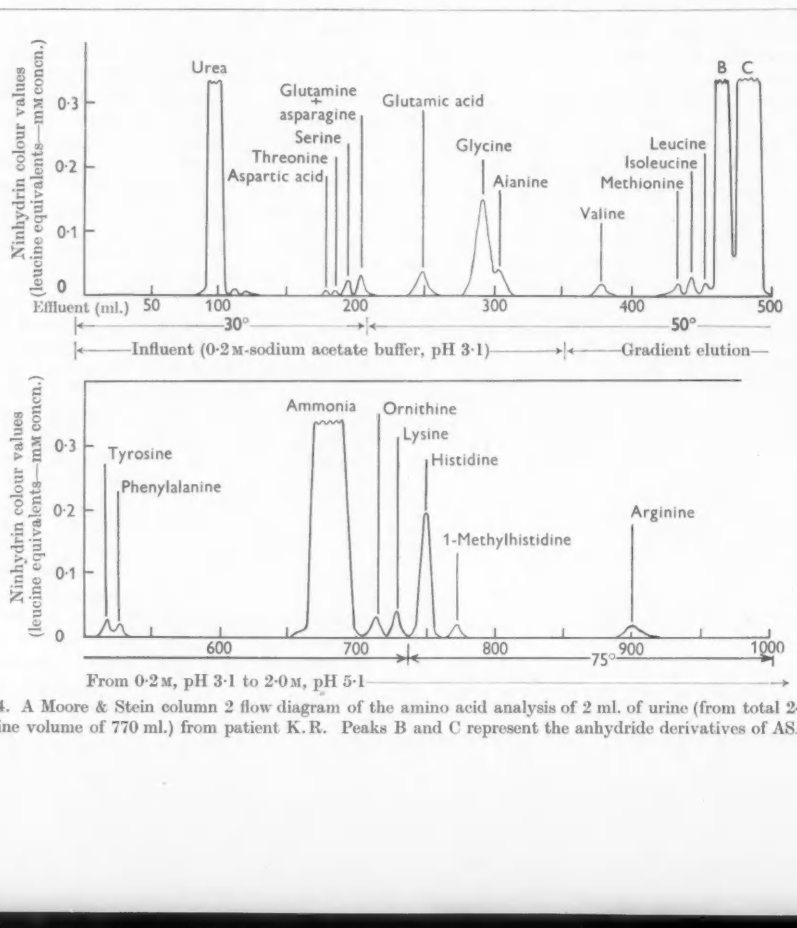


Fig. 4. A Moore & Stein column 2 flow diagram of the amino acid analysis of 2 ml. of urine (from total 24 hr. urine volume of 770 ml.) from patient K.R. Peaks B and C represent the anhydride derivatives of ASA.

Table 1. *Urinary excretion of argininosuccinic acid and urea of a patient with argininosuccinic aciduria*

Diet/day	Total N in diet (g.)	ASA (g./24 hr.)	Urea (g./24 hr.)	ASA N + urea N (g./24 hr.)	ASA N as % of total N
30 g. of protein	4·8	3·1*	7·1*	3·9	14
30 g. of protein + 2 g. of arginine	5·4	2·8	6·4	3·5	13·5
30 g. of protein + 5 g. of aspartic acid	5·3	2·6	5·4	3·0	15
30 g. of protein + 4 g. of ornithine	5·6	3·8	7·0	4·0	16·5
30 g. of protein + 4 g. of citrulline	5·7	4·2	5·2	3·2	22·5
10 g. of protein	1·6	2·7†	4·5†	2·6	18·0

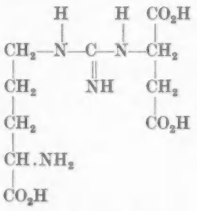
* Mean of 3 days. † Mean of 5 days.

(as above) by the Moore & Stein method and urea by the standard hypobromite method. No firm conclusions can be drawn from a trial of this nature where the tests were made on one child only and were of short duration. However, from the results obtained it would appear that the output of ASA in the urine was little affected by the amount of protein in the diet as on the low protein intake he was still excreting 2·7 g./day, although he must have been in negative N balance as the urine N was greater than dietary N. In view of this, one would not expect additional arginine to increase the output of ASA and this in fact was the case. On the other hand, ornithine and citrulline, which are not found in appreciable quantities in normal diets, and which in the Krebs–Henseleit urea cycle modified by Ratner & Pappas (1949) are nearer precursors of ASA, increased the output of ASA quite appreciably.

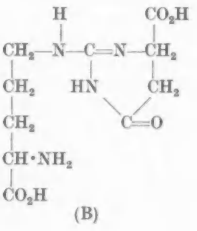
DISCUSSION

In view of the chemical evidence that has been presented, coupled with the paper-chromatographic and paper-electrophoretic comparisons with authentic samples, there seems little doubt that the unknown substance excreted by the patient studied is argininosuccinic acid (ASA). Its complicated behaviour in solution is, however, worthy of further comment. In discussing this, Ratner *et al.* (1953) postulated that of the several forms of cyclization that are possible the two forms shown opposite were the most likely (B, C).

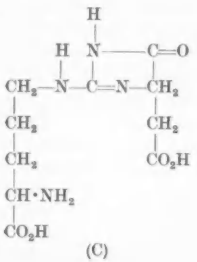
Ratner and her colleagues concluded that they were dealing with only one of these two forms and, since it is difficult, analytically, to differentiate between them, they could not definitely decide which form they had. However, since their anhydride gave a Jaffé reaction by analogy with creatinine, and since, with an isoelectric point at pH 5·5, it would seem that the stronger of the two carboxyl groups of the succinic acid moiety is involved in the cyclization, it is probable that their anhydride was the five-membered-ring form. Comparative tests have shown that substance C is identical with Ratner's anhydride and it has been assumed provisionally in the absence of absolute proof that C has the five-membered-ring structure. With the same reservations substance B has been allocated the six-membered-ring structure. It has an isoelectric point at pH 4·2, which might be expected if the weaker carboxyl group of the succinic acid moiety (i.e. the γ-carboxyl of aspartic acid) was involved in the cyclization. It also fails to give a Jaffé reaction or any colour with the creatinine reagent of Van Pilsum, Martin, Kito & Hess (1956), but it will give a weak blue colour with the alkaline nitroprusside–ferricyanide reagent (Kirby-Berry *et al.* 1951).



(Argininosuccinic acid)



(B)



(C)

Both cyclic forms, apart from some conversion of B into C, are almost completely stable to acid hydrolysis. On the other hand, in alkaline conditions the rings are opened, more slowly with B than with C, to regenerate ASA, which is then susceptible to further breakdown. The degradation is complex and presumably is related to the resonating effect of the double bond, which causes a variation in the site that is the weakest link in the chain. Arginine and citrulline have both been detected after mild alkaline hydrolyses but the formation of arginine by hydrolysis would also yield malic acid, which ultimately would affect the final yield of aspartic acid. Ratner *et al.* (1953), however, found that ASA, after strong alkaline hydrolysis, gave ornithine and aspartic acid in close to theoretical yield. To explain this it must either be postulated that the mechanism of breakdown is different under strong alkaline conditions or that the cleavage to form arginine is not hydrolytic and that direct cleavage forms fumaric acid, which later reacts with free NH_3 to form aspartic acid. The formation of aspartic acid from fumaric acid and ammonium carbonate heated in aqueous solution in a closed tube can readily be demonstrated.

With regard to the biological significance of these curious substances derived from ASA, it may well be that they are only chemical artifacts which have no place in the metabolism of ASA. Ratner *et al.* (1953) found that the anhydride form C was inert in various enzyme systems. Although B has so far not been tested, it is potentially the more interesting substance since, as Ratner (1954) has observed, it can be regarded as a substituted dihydro-orotic acid, a substance which has been shown to be on the pathway to pyrimidine synthesis (Wright *et al.* 1951).

In addition to the chemical problems, believed to concern cyclization of ASA as mentioned above, there still remains the problem of the unidentified substances designated Y and Z. It is now established that Z is not a chemical degradation product of ASA, as was thought earlier, but is derived from Y, which was present in the crude barium argininosuccinate preparation. The possibility still remained that Y might be an artifact formed by procedures employed for the isolation of ASA. However, it was detected on electrophoresis diagrams (by the use of the alkaline nitroprusside–ferricyanide reagent) of fresh urine specimens from the patient excreting large amounts of ASA. It was not present in a number of normal urines, including those from the unaffected members of the patient's own family. Hence it would seem reasonable to assume that Y is a naturally occurring metabolite that, like ASA itself, is normally an intracellular substance which is only rarely excreted in the urine. With this assumption and with the chemical evidence that Y must have a structure somewhat similar to that of ASA (since it gives the same final degradation products, namely, aspartic acid and ornithine), it can be speculated that Y may be another intermediate in the series of reactions which make up the Krebs–Henseleit urea cycle. On the other hand, Y may represent an attempt by the patient to conjugate some of the excess of ASA formed in the tissues and to produce a derivative which is less harmful and better tolerated. It is hoped that when both Y and Z are fully characterized it will be possible to explain their function.

In considering the pathogenesis of the clinical syndrome in the two affected children we are in some difficulty because of inadequate data. At the moment no other similar patients have been described and enough family data is therefore lacking. Nevertheless, it seems probable that the disease is hereditary and probably due to some enzyme defect, similar to those already found in other metabolic diseases, e.g. phenylketonuria, galactosaemia, etc. On considering the urea cycle, Ratner *et al.* (1953) have shown that an enzyme (argininosuccinase) occurs in the liver and catalyses the breakdown of ASA with the formation of arginine and fumaric acid. One might expect therefore that the genetic defect might be a deficiency in this enzyme. If our current ideas are correct, this should lead to an inability to form urea whereas Allan *et al.* (1958) showed that in the disease the ability to form urea seemed unimpaired. Hence it is difficult to believe that the splitting enzyme is absent in the liver, which is regarded as the seat of urea production.

The second unusual feature is that the concentration of ASA is nearly three times as high in the cerebrospinal fluid as in the systemic blood plasma (Allan *et al.* 1958). This was a very striking finding since most of the amino acids found in cerebrospinal fluid are present in much higher concentration in plasma. This makes it most unlikely that the cerebrospinal fluid derives its ASA from the plasma, and in any case ASA has poor permeability properties (Walker & Myers, 1953) and could hardly be expected to penetrate the blood–brain barrier. Thus at present there is no better suggestion than that already discussed by Allen *et al.* (1958) in which they speculate that the unknown metabolite (ASA) may be formed in the brain, or in some other structure closely associated with the cerebrospinal fluid, and leaks from there to the blood, from which it is rapidly cleared by the kidney. This suggestion is not as bizarre as it might seem. For years it has been assumed that urea is formed only in the liver and that its presence in the cerebrospinal fluid was due to infiltration from the blood plasma. Recent work by Sporn, Dingman,

Defalco & Davies (1959) has shown that rat brain is capable of urea synthesis *in vivo* and Walker (1958) has shown that argininosuccinase (splitting enzyme), previously found in mammals only in the liver and kidney (Ratner, 1954), is also present in various other organs of the dog, including the brain.

The feeding experiments were undertaken to see how far the excretion of ASA could be influenced by the diet. In this way it was hoped that some indication might be given which would lead to a rational therapy which would lessen the synthesis of ASA and lower its concentration in the body fluids. If, for instance, arginine had increased the excretion of ASA, then it would be reasonable to attempt to lower the intake of arginine. However, the only compounds found to increase ASA output were ornithine and citrulline, substances not found as such in appreciable quantities in the normal diet. At the moment therefore it is difficult to see what dietary therapy can be usefully tried.

The increased output of ASA after taking additional ornithine and citrulline serves as additional evidence for its identification, and for its close relationship metabolically with these compounds. The effect of a higher protein diet was not tried since the child seemed disinclined to eat more protein than was given in the 30 g./day regimen.

EXPERIMENTAL

Isolation of barium argininosuccinate. Urine (25 l.) from case 2 (Allan *et al.* 1958) was decreased in volume to 4 l. in a rotating evaporator (Van Heyningen, 1949). A hot saturated solution containing 350 g. of $Ba(OH)_2,8H_2O$ was added with stirring and the mixture was allowed to stand overnight. The precipitate was removed by filtration and to the filtrate (5·4 l.) 3 vol. of ethanol and 50 g. of solid $Ba(OH)_2,8H_2O$ were added. After standing for 4 hr. the precipitate was separated by centrifuging, transferred to a large Büchner funnel and washed with 75% ethanol. After a final washing with 95% ethanol the solid cake was broken up and allowed to dry at room temperature. The powder was finally dried in a desiccator over H_2SO_4. Yield of crude barium argininosuccinate was 160 g. Two-way paper-chromatographic analysis for amino acids with phenol–NH_3 and lutidine–water (2·2:1, v/v) as solvents and 25 μg. of the sample showed a large spot, due to ASA, and weak spots, due to traces of aspartic acid and glutamic acid. The crude barium salt (60 g.) was dissolved in 200 ml. of water and an insoluble residue was discarded. Ethanol (600 ml.) was added to the clear solution and the reprecipitated barium salt was filtered and dried. The yield was 47 g. Paper-chromatographic analysis showed ASA as the only ninhydrin-reacting constituent.

Preparation of free argininosuccinic acid (1). The purified barium salt of ASA (5 g.) was dissolved in 25 ml. of water and the small insoluble residue was discarded. The clear solution was chilled (4°) and N-H_2SO_4 (19 ml.), also cooled, was added to bring the pH of the mixture to 3·4. At the exact end point no precipitate was obtained when either more acid or weak $Ba(OH)_2$ solution was added to a centrifuged portion of the mixture. The $BaSO_4$ was removed by centrifuging and the clear solution was freeze-dried. A fluffy pale-cream-coloured powder was obtained. Yield was 2·4 g. (Found: N, 18·9. Theory: N, 19·3%).

Preparation of free argininosuccinic acid (2). A glass tube 15 cm. long and 2·4 cm. in diameter constricted to capillary bore at the lower end and plugged with glass wool was filled with Dowex-50 cation-exchange resin (4% cross-linked and 100–200 mesh; 7·5 g. dry wt.). The resin was packed as a slurry after pretreatment with 2N-HCl and washing with water by decantation. Aq. N-NH_3 soln. was run down the column until the effluent was strongly alkaline. The column was then washed with water to remove the excess of NH_3 and kept at 4° until required. The purified barium salt of ASA (5 g.) was dissolved in 40 ml. of water and a small insoluble residue discarded. The solution was chilled and then passed through the prepared resin column. The effluent containing the soluble ammonium salt of ASA was collected and freeze-dried. Final drying was carried out in a Fischer drier at 80°. Yield was 2·1 g. (Found: N, 19·1. Theory: N, 19·3%).

Preparation of argininosuccinic acid anhydride (*substance* C). Free ASA (1·5 g.) was dissolved in 10 ml. of water and was allowed to stand at room temperature for 2 days. Ethanol (10 ml.) was added and as soon as crystals appeared a further 20 ml. of ethanol was added gradually. After standing at 4° overnight the crop of crystals was filtered off and dried. Yield 0·7 g. [Found: C, 44·1; H, 6·10; N, 20·4 (Dumas). $C_{10}H_{16}O_5N_4$ requires C, 44·1; H, 5·92; N, 20·6%].

Isolation of substances derived from partial degradation of crude argininosuccinic acid with alkali

The crude barium salt of ASA (10 g.) was dissoved in 100 ml. of water and 2N-H_2SO_4 was added cautiously until the mixture was adjusted to pH 3·4. $BaSO_4$ was removed by centrifuging and the supernatant was mixed with an equal volume of N-NaOH and refluxed for 4 hr. The hydrolysate was cooled and diluted to 1 l. with water and then run through a resin-column system (Westall, 1952), consisting of two columns coupled one beneath the other, filled with the anion-exchange resin Dowex-2 (8% cross-linked and 100–200 mesh). The upper column was made from a filtration tube 1·7 cm. in diameter and having a resin bed 12 cm. long (containing 10 g. of dry resin). The second column had a tube 1·2 cm. in diameter with a resin bed 8 cm. high (3·5 g. of dry resin). The tubes were packed with a slurry of resin in water and then coupled together with capillary glass tubing. The resin was converted into the OH form by passing 2N-NaOH down the columns followed by washing with water until the pH of the effluent was between 7 and 8. After the diluted hydrolysate was passed down the columns a further 100 ml. of water was run on and this was followed by the displacing agent 0·1N-HCl. As soon as a positive ninhydrin reaction was obtained on testing the effluent, fractions (8–10 ml.) were collected until the effluent became strongly acid. Samples (5 μl.) from the fractions were spotted on paper strips and the strips were analysed by electrophoresis. The amino acid composition of the fractions is illustrated in Fig. 3.

Isolation of ornithine hydrochloride. Fractions 1–11 were mixed, adjusted to pH 6 with N-HCl and evaporated at reduced pressure to 5 ml. Crystals were obtained after the gradual addition of ethanol to give a final volume of 20 ml.

The crystals were collected and recrystallized from water and ethanol. Yield was 0·82 g. (Found: C, 35·8; H, 7·1; N, 16·7. $C_5H_{13}O_2N_2Cl$ requires C, 35·6; H, 7·7; N, 16·6%).

Isolation of citrulline. Fractions 13–16 were combined and evaporated under reduced pressure to 2 ml. Crystals were obtained after the addition of ethanol. Yield after recrystallization was 0·21 g. (Found: C, 41·6; H, 7·7; N, 23·8. $C_6H_{13}O_3N_3$ requires C, 41·1; H, 7·5; N, 24·0%).

Isolation of substance B. Fractions 17–19 were mixed and evaporated under reduced pressure to 2 ml. A crystalline product was obtained by the addition of ethanol. Yield was 0·14 g. This material gave a lower figure for its N content than was expected and it was ultimately discovered that it was contaminated with an unknown, non-ninhydrin-reacting ampholyte which had a lower N content. A pure sample of B was obtained later by repeated recrystallization, from aqueous ethanol, of a sample obtained from a larger-scale fractionation carried out in a similar manner. Yield was 1·7 g. [Found: C, 43·9; H, 6·1; N, 20·8 (Dumas). $C_{10}H_{16}O_5N_4$ requires C, 44·1; H, 5·9; N, 20·6%].

Isolation of substance Z. Substance Z formed a white deposit shortly after the fractions were collected. This white powder was filtered off from fractions 22–26 and the fractions were decreased in volume to 10 ml. and a further crop was obtained. This powder was very slightly soluble in water and was washed well with cold water before drying. Yield was 0·32 g. (Found: C, 44·61; H, 5·46; N, 15·3%).

Isolation of aspartic acid. Small amounts of aspartic acid crystallized spontaneously in fraction tubes 28–32. This material was filtered off and a second crop was obtained by decreasing the volume of the mother liquor to 5 ml. Both crops were combined and recrystallized from hot water. Yield was 0·21 g. (Found: C, 36·37; H, 5·33; N, 10·70. $C_4H_7O_4N$ requires C, 36·1; H, 5·3; N, 10·52%).

SUMMARY

1. The unknown metabolite previously described as the predominant amino acid in the urine of two mentally defective sibs has been isolated and has proved to be argininosuccinic acid.

2. The behaviour of argininosuccinic acid in aqueous solution has been studied. It apparently forms an equilibrium mixture with two other modified forms which are probably cyclized anhydrides.

3. The complicated behaviour of argininosuccinic acid on resin columns can be explained on the basis of its known reactivity. It is, nevertheless, possible to assay it by the method of Moore & Stein (1954). Each patient with argininosuccinic aciduria excreted about 3 g. of argininosuccinic acid per 24 hr. in his urine.

4. Argininosuccinic acid and its two anhydride forms yield ornithine and aspartic acid after long hydrolysis with strong alkali, but with weaker alkali for a shorter time a number of intermediates are formed which include arginine and citrulline.

5. An unknown substance has been found in the urine of one patient with argininosuccinic aciduria which after treatment with strong alkali breaks down to yield aspartic acid and ornithine.

6. The gross excretion of argininosuccinic acid seemed little affected by lowering the protein content of the diet over a short period or by adding arginine, but it was increased by feeding ornithine and citrulline.

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REFERENCES

Allan, J. D., Cusworth, D. C., Dent, C. E. & Wilson, V. K. (1958). *Lancet*, i, 182.
Cusworth, D. C. & Dent, C. E. (1960). *Biochem. J.* **74**, 550.
Davison, D. C. & Elliott, W. H. (1952). *Nature, Lond.*, **169**, 313.
Dent, C. E. (1951). *Recent Advances in Clinical Pathology*, 2nd ed., p. 238. London: J. and A. Churchill Ltd.
Fincham, J. R. S. & Boylen, J. B. (1955). *Biochem. J.* **61**, xxiii.
Gross, D. (1955). *Nature, Lond.*, **176**, 72.
Kirby-Berry, H., Sutton, H. E., Cain, L. & Berry, J. S. (1951). *Texas Univ. Publ.* No. 5509, 22.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
Ratner, S. (1954). *Advanc. Enzymol.* **15**, 319, 357.
Ratner, S. & Pappas, A. (1949). *J. biol. Chem.* **179**, 1183.
Ratner, S., Petrack, B. & Rochovansky, O. (1953). *J. biol. Chem.* **204**, 95.
Sporn, M. B., Dingman, W., Defalco, A. & Davies, R. K. (1959). *Nature, Lond.*, **183**, 1520.
Theorell, T. & Stenhagen, E. (1939). *Biochem. Z.* **299**, 417.
Van Heyningen, W. E. (1949). *Brit. J. exp. Path.* **30**, 302.
Van Pilsum, J. F., Martin, R. P., Kito, E. & Hess, J. (1956). *J. biol. Chem.* **222**, 225.
Walker, J. B. (1958). *Proc. Soc. exp. Biol., N.Y.*, **98**, 7.
Walker, J. B. & Myers, J. (1953). *J. biol. Chem.* **203**, 143.
Westall, R. G. (1952). *Biochem. J.* **52**, 638.
Westall, R. G. (1955). *Biochem. J.* **60**, 247.
Westall, R. G. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 13–34, p. 168.
Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L. & Wilson, D. W. (1951). *J. Amer. chem. Soc.* **73**, 1898.

Studies on Pyridine Nucleotide Biosynthesis

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Until recently it had been generally accepted that the formation of diphosphopyridine nucleotide in biological systems starts from nicotinamide, with nicotinamide riboside and nicotinamide mononucleotide as intermediates (Singer & Kearney, 1954). Much of the earlier work on whether nicotinic acid or nicotinamide is the more efficient precursor of diphosphopyridine nucleotide has been controversial, but Preiss & Handler (1958*a*, *b*) unequivocally demonstrated that biosynthesis of diphosphopyridine nucleotide could also proceed by a mechanism alternative to the nicotinamide pathway referred to above, as follows:

Nicotinic acid + 5-phosphoribose pyrophosphate →
deamido nicotinamide mononucleotide + pyrophosphate

Deamido nicotinamide mononucleotide + adenosine triphosphate →
deamido diphosphopyridine nucleotide + pyrophosphate

Deamido diphosphopyridine nucleotide + glutamine + adenosine triphosphate →
diphosphopyridine nucleotide + glutamate + adenosine monophosphate + pyrophosphate.

It is known that micro-organisms (Hughes & Williamson, 1953; Oka, 1954; Halpern & Grossowicz, 1957; Sundaram, Rajagopalan & Sarma, 1958), the insect *Corcyra cephalonica* St. (Sundaram, Rajagopalan & Sarma, 1959; Sundaram, Radhakrishnamurty & Sarma, 1954) and the pigeon and the chick (Rajagopalan, Sundaram & Sarma, 1958) possess the enzyme nicotinamide deamidase. Consequently, nicotinic acid is an important end product of metabolism of nicotinamide in these organisms. With the demonstration of the nicotinic acid pathway for biosynthesis of diphosphopyridine nucleotide, it appeared likely that nicotinamide deamidase might function not only by converting nicotinamide into nicotinic acid as an end product but also as a means whereby the free nicotinic acid needed for biosynthesis of diphosphopyridine nucleotide might be formed from nicotinamide. The work reported in this paper relates to the biosynthesis of diphosphopyridine nucleotide in *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides* 9135.

EXPERIMENTAL

Materials

The strain of *Saccharomyces cerevisiae* used was maintained by fortnightly subculture on a malt–agar medium. For growth experiments cells from a 24 hr. culture were used for seeding. *Leuconostoc mesenteroides* 9135, kindly supplied by Dr B. C. Johnson, University of Illinois, Urbana, was maintained by fortnightly subculture on a medium containing, in 100 ml.: glucose, 1 g.; sodium acetate, 2 g.; Difco yeast extract, 0·5 g.; liver extract, 0·6 g.; peptone, 0·5 g.; K_2HPO_4, 50 mg.; KH_2PO_4, 50 mg.; $MgSO_4.7H_2O$, 20 mg.; NaCl, 1·0 mg.; $MnSO_4.4H_2O$, 1·0 mg.; $FeSO_4.7H_2O$, 1·0 mg.; agar, 2 g.; the pH was adjusted to 6·8–7·0.

N'-Methylnicotinamide was a gift from Dr H. P. Sarett. [*carboxy*-^{14}C]Nicotinic acid and [*carboxy*-^{14}C]nicotinamide were obtained through the courtesy of Dr B. C. Johnson.

Methods

Metabolic products of nicotinic acid and nicotinamide in growing cultures of Saccharomyces cerevisiae. *S. cerevisiae* was grown for 18 hr. on the medium of Atkin, Williams, Schultz & Frey (1950) supplemented with nicotinic acid or nicotinamide at 10 μg./ml. The culture medium, after concentration, was analysed for metabolites by paper chromatography as described by Sundaram *et al.* (1958).

Deamidation of nicotinamide by Saccharomyces cerevisiae *and* Leuconostoc mesenteroides. Cells of *S. cerevisiae*, grown on the above described medium, and of *L. mesenteroides* grown on the medium of Krehl, De La Huerga, Elvehjem & Hart (1946) supplemented with nicotinic acid (0·25 μg./ml.), were harvested, washed with aqueous 0·9% NaCl by centrifuging and dispersed in this medium to give a suspension containing 200 mg. of cells/ml. The suspensions were assayed for nicotinamide deamidase by the microbiological method described by Sundaram *et al.* (1958). Cell-free extracts of *S. cerevisiae* and *L. mesenteroides*

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were obtained by grinding the frozen cells with sand in a chilled mortar, extracting the ground mass with ice-cold water and centrifuging the slurry to sediment sand and cell debris.

Biosynthesis of diphosphopyridine nucleotide by cultures of Saccharomyces cerevisiae. *S. cerevisiae* was grown for 18 hr. on the basal medium supplemented, where necessary, with nicotinic acid or nicotinamide. The cells were harvested and washed with 0·9% NaCl by centrifuging at 3000 *g* for 15 min. and assayed for pyridine nucleotides by the following method. The cells were treated with 5% (w/v) trichloroacetic acid and homogenized for 2 min. in a Potter–Elvehjem homogenizer with a Teflon pestle (A. H. Thomas Co., Philadelphia, Pa., U.S.A.). The homogenate was centrifuged at 8000 *g* for 10 min. and the supernatant, after suitable dilution with 5% trichloroacetic acid, was assayed by the fluorimetric method of Carpenter & Kodicek (1950) with butan-2-one; *N'*-methylnicotinamide was the internal standard. Under these conditions, *S. cerevisiae* does not accumulate *N'*-methylnicotinamide as a metabolite of either nicotinic acid or nicotinamide. Hence the value obtained in the fluorimetric method was taken to represent diphosphopyridine nucleotide (DPN) (see below).

The culture medium of *S. cerevisiae*, after harvesting of cells, was steamed for 15 min. and assayed microbiologically for nicotinic acid plus nicotinamide and for nicotinic acid alone with *Lactobacillus arabinosus* 17–5 and *Leuconostoc mesenteroides* 9135 respectively (Snell & György, 1950).

Biosynthesis of diphosphopyridine nucleotide by suspensions of Saccharomyces cerevisiae *and* Leuconostoc mesenteroides. Cells of *S. cerevisiae* grown on the unsupplemented basal medium were harvested by centrifuging at 3000 *g* for 15 min., washed three times with sterile 0·9% NaCl by centrifuging and suspended in sterile water. *L. mesenteroides* was grown on the medium of Krehl *et al.* (1946) supplemented with suboptimum amounts of nicotinic acid (10 μg./ml.), harvested, washed with sterile 0·9% NaCl and suspended in sterile water as for *S. cerevisiae*. The incubation system contained: 31 mM-glucose; 5·3 mM-glutamine; 12·5 mM-KH_2PO_4 (K_2HPO_4 with *L. mesenteroides*); 2 μmoles of nicotinic acid or nicotinamide were added where necessary and 1 g. fresh wt. of the organism in a final volume of 5 ml.; KCl was added to make the medium equivalent to a 0·152 M solution of di-ionic electrolyte. All the reagents were sterilized at 10 lb./in.^{2} for 10 min. before use. Incubation was in 50 ml. conical flasks plugged with sterilized cotton wool in a mechanical shaker at 30° for 18–20 hr. The incubation mixture was then treated with 5% (w/v) trichloroacetic acid and homogenized. The homogenate was centrifuged at 8000 *g* for 10 min. and the supernatant, after suitable dilution with 5% trichloroacetic acid, was assayed for pyridine nucleotides. The experiments on pyridine nucleotide synthesis were repeated seven or eight times. The same pattern of results was observed throughout, though with minor quantitative differences. The deamidation of nicotinamide was also followed in these experiments with *S. cerevisiae*. Samples of the mixtures which contained nicotinamide were withdrawn at intervals of 1, 2 and 20 hr., heated in a boiling-water bath for 10 min. and assayed for nicotinic acid with *L. mesenteroides*.

Experiments with [^{14}C]*nicotinic acid and* [^{14}C]*nicotinamide.* The experimental conditions were similar to those in the previous experiment, except that the incubation mixture, where necessary, contained 2 μmoles of ^{14}C-labelled nicotinic acid or nicotinamide (1 μc). After incubation, trichloroacetic acid was added to a final concentration of 5% and the mixture was homogenized. A portion of the supernatant obtained by centrifuging at 8000 *g* was taken for estimation of pyridine nucleotides. Another portion was extracted with peroxide-free ether to remove trichloroacetic acid, evaporated to dryness in a vacuum desiccator and taken up in a small volume of water. After centrifuging, the supernatant was chromatographed with 95% ethanol–M-ammonium acetate (7:3, v/v), the solution having been adjusted to pH 5·0 with HCl, for 20 hr. on Whatman no. 1 paper alongside reference standards of nicotinic acid, nicotinamide and DPN. The chromatogram was air-dried and cut into 1 cm. segments along the direction of flow of the solvent, and radioactivity in the segments was measured in stainless-steel planchets by using a Geiger counter tube with mica window (thickness 2·1 mg./cm.^{2}) connected to a Panax scaler (Panax Equipment Co., Mitcham, Surrey). A second chromatogram was radioautographed on Ilford Ilfex X-ray film, the period of exposure being 4 weeks.

RESULTS

Metabolic products of nicotinic acid and nicotinamide formed by Saccharomyces cerevisiae. Analyses of the culture medium of the organism by paper chromatography revealed that nicotinic acid accumulated as the chief metabolic end product. *N'*-Methylnicotinamide and nicotinuric acid were absent and where nicotinamide had been the supplement it was almost completely deamidated.

Deamidation of nicotinamide by Saccharomyces cerevisiae *and* Leuconostoc mesenteroides. The data in Table 1 show the extent of deamidation of nicotinamide by cell extracts and resting cells of *S. cerevisiae* and *L. mesenteroides*; suspensions of *S. cerevisiae* as well as cell-free extract, prepared by grinding the cells with sand, had deamidase activity. On the other hand, *L. mesenteroides* 9135, which can grow only in the presence of nicotinic acid (Johnson, 1945) was devoid of this enzyme.

Table 1. *Deamidation of nicotinamide by resting cells and cell extracts of* Saccharomyces cerevisiae *and* Leuconostoc mesenteroides 9135

Washed fresh cells (100 mg.) were incubated with 6 μmoles of nicotinamide in 2 ml. of 0·1 M-phosphate buffer, pH 7·5, for 1 hr. With cell extract, 0·5 ml. of 10% (w/v) extract of fresh cells of *S. cerevisiae* was similarly incubated, in a final volume of 2 ml. The free nicotinic acid was estimated microbiologically with *L. mesenteroides* 9135.

System	Nicotinic acid released (μm-moles/hr.)
S. cerevisiae, resting cells	1120
L. mesenteroides, resting cells	1·4
S. cerevisiae, cell extract	430*
L. mesenteroides, cell extract	Nil

* Amount/mg. of extract nitrogen.

Biosynthesis of diphosphopyridine nucleotide by cultures of Saccharomyces cerevisiae. *S. cerevisiae* synthesizes DPN equally well from nicotinic acid and nicotinamide (Table 2). The analysis of the culture medium shows that the organism rapidly metabolizes nicotinamide to nicotinic acid, which is the only form of the vitamin finally present in the medium. Little nicotinamide would therefore be available for conversion into DPN by the nicotinamide pathway; since the synthesis of DPN is about the same whether nicotinic acid or nicotinamide has been the original supplement it is reasonable to infer that the nicotinic acid pathway is normally used for the coenzyme synthesis in *S. cerevisiae*. Paper chromatography and paper electrophoresis of the pyridine nucleotide-containing extracts revealed the presence of only one quaternary nicotinamide derivative corresponding to DPN. No spot corresponding to nicotinamide mononucleotide was detected. Thus what is being measured by the fluorimetric method is DPN.

Biosynthesis of diphosphopyridine nucleotide by resting cells of Saccharomyces cerevisiae *and* Leuconostoc mesenteroides 9135. Suspensions of both *S. cerevisiae* and *L. mesenteroides* synthesize DPN when the vitamin is supplied in the acid form (Table 3). Nicotinamide is about equally efficient as a precursor of DPN in *S. cerevisiae*, whereas in

Table 2. *Biosynthesis of diphosphopyridine nucleotide and deamidation of nicotinamide by cultures of* Saccharomyces cerevisiae

S. cerevisiae was grown for 20 hr. on medium (100 ml.) supplemented where necessary with 1 mg. of nicotinic acid or nicotinamide. The cells were harvested and assayed for diphosphopyridine nucleotide and the culture medium was analysed microbiologically for nicotinic acid with *L. mesenteroides* and for nicotinic acid + nicotinamide (if any) with *Lactobacillus arabinosus*.

Supplement to growth medium	Fresh wt. of cells (g.)	DPN content (μg.)	DPN synthesized (μg.)	Nicotinic acid in culture medium (μg.)	
				L. arabinosus	*L. mesenteroides*
Nil	1·7	306	—	—	—
Nicotinic acid	1·7	759	453	737	750
Nicotinamide	1·6	759	453	827	824

Table 3. *Biosynthesis of diphosphopyridine nucleotide by suspensions of* Saccharomyces cerevisiae *and* Leuconostoc mesenteroides

Suspensions of *S. cerevisiae* and *L. mesenteroides* were incubated as described in the Experimental section, with supplement, where necessary, of nicotinic acid or nicotinamide (2 μmoles in 5 ml.). The incubation mixture was treated with trichloroacetic acid and assayed for diphosphopyridine nucleotide.

Organism	Supplement to incubation mixture	DPN content (μmoles/g. fresh wt. of cells)	DPN synthesis (μmoles/g. fresh wt. of cells)
S. cerevisiae	Nil	0·37	—
	Nicotinic acid	0·89	0·52
	Nicotinamide	0·83	0·46
L. mesenteroides	Nil	0·22	—
	Nicotinic acid	0·47	0·25
	Nicotinamide	0·23	0·01

Table 4. *Deamidation of nicotinamide by suspensions of* Saccharomyces cerevisiae *under conditions of diphosphopyridine nucleotide biosynthesis*

Suspensions of *S. cerevisiae* were incubated as described in Table 3 with supplement where necessary of nicotinamide (250 μg./5 ml.). At intervals of 1, 2 and 20 hr. portions of mixture were withdrawn, heated in a boiling-water bath and assayed for nicotinic acid.

Supplement	Time (hr.)	Nicotinic acid in 5 ml. of incubation mixture (μg.)	Nicotinic acid formed by deamidation of nicotinamide* (μg., by difference)
None	1	0	214
Nicotinamide	1	214	
None	2	8	205
Nicotinamide	2	213	
None	20	35	255
Nicotinamide	20	290	

* Vitamin utilized for diphosphopyridine nucleotide synthesis is not included in these figures.

L. mesenteroides there is practically no synthesis from the amide. It is reasonable to presume that *L. mesenteroides* adopts exclusively the nicotinic acid pathway for the synthesis of DPN because there is no nicotinamide deamidase in this organism. This deficiency is also reflected in the inability of nicotinamide to support the growth of *L. mesenteroides*. *S. cerevisiae* utilizes nicotinamide as well as nicotinic acid for the biosynthesis of DPN. The deamidation of nicotinamide is rapid under the conditions used, most of the amide being converted into the acid within 1 hr. (Table 4). This supports the conclusion that *S. cerevisiae* utilizes nicotinic acid normally for biosynthesis and that when nicotinamide is the form of the vitamin provided, the organism rapidly deamidates it and metabolizes it to DPN by the nicotinic acid pathway.

Experiments with [^{14}C]*nicotinic acid and* [^{14}C]*nicotinamide.* Figs. 1 (*a*) and (*b*) show that the radioactivity on the chromatograms is mainly associated with nicotinic acid, confirming a rapid deamidation of nicotinamide by *S. cerevisiae*. Moreover, the radioactivity associated with the DPN spot is of the same order of magnitude in both, suggesting that nicotinamide is used as efficiently as nicotinic acid for biosynthesis of DPN. The identity of the material in spot B could not be determined. If this spot represents one of the intermediates in DPN synthesis, such as a riboside or a ribotide, the fact that the spot did not fluoresce on exposure to ammonia–butan-2-one would indicate that it is a derivative of nicotinic acid and not of the amide (Preiss & Handler, 1958*a*).

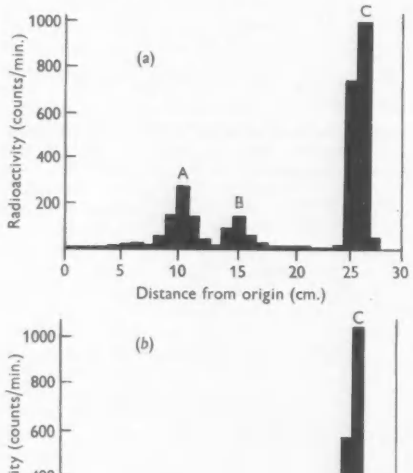

Fig. 1. Formation of pyridine nucleotides from [^{14}C]-nicotinic acid (*a*) and [^{14}C]nicotinamide (*b*) by suspensions of *S. cerevisiae*. Radioautographs of incubation system supplemented with [^{14}C]nicotinic acid and [^{14}C]nicotinamide. A, DPN; B, unknown; C, nicotinic acid. Solvent system: 95% ethanol–M-ammonium acetate, pH 5·0 (7:3).

DISCUSSION

Preiss & Handler (1958*b*) established that yeast, like human erythrocytes, possesses enzymes for synthesizing pyridine nucleotides from nicotinic acid via the nicotinic acid pathway and that yeast autolysates formed the pyridine nucleotide coenzymes from nicotinic acid but not from nicotinamide. The present investigation has shown that yeast can synthesize DPN from nicotinamide as readily as from nicotinic acid. This is in accord with the high nicotinamide-deamidating activity of *S. cerevisiae*. Nicotinamide deamidase is also present in beer yeast (Oka, 1954). It is reasonable to conclude therefore that the nicotinamide-deamidating enzyme had been destroyed in the yeast autolysates of the American workers.

Although the enzymes for the synthesis of DPN by the nicotinamide pathway are present in yeast (Hundley, 1954), the results of Preiss & Handler (1958*b*) and of the present study show that it is the nicotinic acid pathway that is normally responsible for the synthesis of DPN in yeast. Similarly, in human erythrocytes, even though the full complement of enzymes of the nicotinamide pathway is available, the nicotinic acid pathway is probably the predominant route for synthesis of DPN (Preiss & Handler, 1958*a*).

In *L. mesenteroides*, an organism devoid of nicotinamide deamidase and utilizing only nicotinic acid for growth, the nicotinic acid pathway accounts for the formation of DPN. This mechanism of the synthesis of DPN seems quite widespread; in organisms in which nicotinamide deamidase is present, it may be the normal route of the formation of DPN.

SUMMARY

1. *Saccharomyces cerevisiae* when grown in the presence of either nicotinic acid or nicotinamide accumulates free nicotinic acid as the chief end product. *N'*-Methylnicotinamide and nicotinuric acid are not formed.

2. Resting cells of *S. cerevisiae* as well as a cell-free extract have nicotinamide-deamidase activity. Cells of *Leuconostoc mesenteroides* 9135 are devoid of the deamidase.

3. *S. cerevisiae* can synthesize diphosphopyridine nucleotide from nicotinamide as well as from nicotinic acid and can rapidly deamidate nicotinamide under both growing and resting conditions. Cells of *L. mesenteroides* form diphosphopyridine nucleotide from nicotinic acid but not from nicotinamide.

4. Experiments with [^{14}C]nicotinic acid and [^{14}C]nicotinamide confirm that the biosynthesis of diphosphopyridine nucleotide is effected by suspensions of *S. cerevisiae* almost as efficiently from nicotinamide as from nicotinic acid and that there is a rapid deamidation of nicotinamide.

5. It is concluded that the nicotinic acid pathway of biosynthesis of diphosphopyridine nucleotide is the predominant mechanism under normal conditions in *L. mesenteroides* and *S. cerevisiae*.

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REFERENCES

Atkin, L., Williams, W. L., Schultz, A. S. & Frey, C. N. (1950). Quoted by Snell, E. E. (1950). In *Vitamin Methods*, vol. 1. Ed. by György, P. New York: Academic Press Inc.

Carpenter, K. J. & Kodicek, E. (1950). *Biochem. J.* **46**, 421.

Halpern, Y. S. & Grossowicz, N., (1957). *Biochem. J.* **65**, 716.

Hughes, D. E. & Williamson, D. H. (1953). *Biochem. J.* **55**, 851.

Hundley, J. M. (1954). In *Vitamins*, vol. 11, p. 504. Ed. by Sebrell, W. H., jun. & Harris, R. S. New York: Academic Press Inc.

Johnson, B. C. (1945). *J. biol. Chem.* **159**, 227.

Krehl, W. A., De La Huerga, J., Elvehjem, C. A. & Hart, E. B. (1946). *J. biol. Chem.* **166**, 53.

Oka, Y. (1954). *J. Biochem., Tokyo*, **41**, 89, 107.

Preiss, J. & Handler, P. (1958*a*). *J. biol. Chem.* **233**, 488.

Preiss, J. & Handler, P. (1958*b*). *J. biol. Chem.* **233**, 493.

Rajagopalan, K. V., Sundaram, T. K. & Sarma, P. S. (1958). *Nature, Lond.*, **182**, 51.

Singer, T. P. & Kearney, E. B. (1954). *Advanc. Enzymol.* **15**, 79.

Snell, E. E. & György, P. (1950). In *Vitamin Methods*, vol. 1, p. 360. Ed. by György, P. New York: Academic Press Inc.

Sundaram, T. K., Radhakrishnamurty, R. & Sarma, P. S. (1954). *Curr. Sci.* **23**, 92.

Sundaram, T. K., Rajagopalan, K. V. & Sarma, P. S. (1958). *Biochem. J.* **70**, 196.

Sundaram, T. K., Rajagopalan, K. V. & Sarma, P. S. (1959). *J. Chromat.* **2**, 531.

Biochem. J. (1960) **77**, 149

The Amino Acid Sequence around the Reactive Serine Residue of some Proteolytic Enzymes

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The three endopeptidases of the pancreas, chymotrypsin, trypsin and elastase, are stoicheiometrically inhibited by di*iso*propyl phosphorofluoridate, which combines with an unusually reactive serine residue in the enzymes. In chymotrypsin and trypsin this residue has been shown to be present in the sequence Gly.Asp.Ser.Gly (Turba & Gundlach, 1955; Schaffer, Simet, Harshman, Engle & Drisko, 1957; Oosterbaan, Kunst, Van Rotterdam & Cohen, 1958; Dixon, Kauffman & Neurath, 1958). [For definitions of the abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.] That elastase contains the same sequence was concluded from experiments in which a partial acid hydrolysate of the di*iso*propoxy[^{32}P]phosphinyl derivative of elastase was subjected to paper ionophoresis and shown to contain the same radioactive peptides as did similar hydrolysates of the di*iso*propoxy[^{32}P]-phosphinyl derivatives of chymotrypsin and trypsin (Hartley, Naughton & Sanger, 1959). The present paper describes some further experiments with two-dimensional ionophoresis which confirm the above-mentioned result.

Considerable difficulties were encountered when we attempted to identify the various radioactive peptide spots present in these hydrolysates. The

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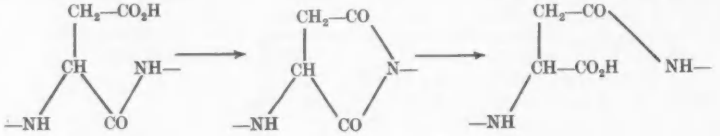
mixture was much more complex than would have been expected and the results could not be explained on the simple assumption that the only reaction taking place during the partial hydrolysis of a protein by acid was the splitting of peptide and amide bonds. Thus, for instance, when hydrolysis was carried out for 3 days in 12N-hydrochloric acid, at least three peptides were present which appeared to have the structure aspartyl–serine phosphate (Asp.SerP) and were interconvertible on further hydrolysis. Another three appeared to have the structure Asp.SerP.Gly. Since the serine phosphate residue in the radioactive proteins was present as the di*iso*propyl derivative heterogeneity was to be expected if the removal of the *iso*propyl groups by acid was not complete (Schaffer *et al.* 1957), and in fact small amounts of mono*iso*propyl derivatives were detected. The main difficulties, however, appeared to be due to some interconversions of the aspartyl residues and the results could best be explained by assuming that the reactions shown in Scheme 1 were taking place.



α–Aspartyl αβ–Aspartyl β–Aspartyl

Scheme 1

That the reaction in Scheme 1 can occur during acid hydrolysis was shown by Swallow & Abraham (1958), who isolated ε-(αβ-aspartyl)lysine [referred to by them as ε-(aminosuccinyl)lysine] from a partial hydrolysate of bacitracin A. In model experiments they also showed that ε-(α-aspartyl)-lysine and ε-(β-aspartyl)lysine were interconvertible but were both largely converted into the αβ-aspartyl derivative. They found that an αβ-aspartyl derivative was formed from α-(α-aspartyl)-lysine but was not as stable as the ε-(αβ-aspartyl)-lysine. The present results agree with those of Swallow & Abraham, and suggest that the abovementioned reactions will be of general occurrence in experiments where partial acid hydrolysis is used on proteins or peptides containing aspartic acid.

One of the objects of this investigation was to try to develop methods for identifying the amino acid sequence in the vicinity of a known isotopically labelled residue when only small amounts of material are available. Such methods would involve studying the electrophoretic and chromatographic rates of the radioactive peptides produced on partial hydrolysis and the effect of degradations such as acid hydrolysis and the Edman procedure on these peptides.

Thus, for instance, it should be possible to determine the number of residues in a labelled peptide from the products it gives on partial acid hydrolysis. A dipeptide should give only two labelled products, unchanged dipeptide and the free amino acid. A tripeptide in which the labelled residue is terminal would give itself, the amino acid and one new dipeptide. A tripeptide with the labelled residue in the middle should give two dipeptides and so forth. Similarly, some of the interrelationships of the different peptides may be deduced by studying the products produced after removal of the *N*-terminal residues by the Edman procedure. It was considered that the present system, in which a single residue in a known sequence is labelled, would be a good one on which to work out the methods. We have thus studied the effect of partial acid hydrolysis on the various bands in some detail and these results have contributed considerably to their identification.

Where enough material was available we have identified the amino acids present in the peptides after complete hydrolysis and in some cases have determined the *N*-terminal residues by the 2:4-dinitrophenyl technique.

EXPERIMENTAL

The following abbreviations for amino acid residues will be used in this paper: SerP, *O*-phosphoserine; SerIP, mono*iso*propyl phosphoserine; SerDIP, di*iso*propyl-phosphoserine.

Materials

Trypsin and chymotrypsin were crystalline salt-free preparations from Worthington Biochemical Corp. Elastase was prepared by a method to be described (Naughton & Sanger, 1960).

Di*iso*propyl [^{32}P]phosphorofluoridate (DF^{32}P) was a gift from Dr R. Davies and usually contained between 1 and 30 μc of ^{32}P/mg.

*Preparation of di*iso*propoxy*[^{32}P]*phosphinyl enzymes*

Various preparations of the di*iso*propoxy[^{32}P]phosphinyl (formerly 'di*iso*propylphosphoryl') derivatives were made. A satisfactory procedure was as follows.

The enzyme (25 mg.) was dissolved in 5 ml. of 0·1 M-phosphate buffer, pH 8·0, and treated with 0·63 ml. of 0·02 M-DF^{32}P in propan-2-ol. After incubation at room temperature for 3hr. the mixture was dialysed against two changes of 5 l. of 0·01 M-acetic acid at 2° and freeze-dried.

Hydrolysis of diisopropoxy[^{32}P]phosphinyl enzymes and peptides

Three methods of hydrolysis were used: (*a*) hydrolysis with 12 N-HCl at 37°; (*b*) hydrolysis with 5·7 N-HCl at 100° in a boiling-water bath; (*c*) hydrolysis with 5·7 N-HCl at 37°.

In general, where only small amounts of protein or peptides were to be hydrolysed the reaction was carried out in small test tubes with about 0·2 ml. of HCl. The HCl was then removed *in vacuo* over NaOH, water was added and the residue dried off about three times. In some experiments redistilled 5·7 N-HCl was used, but this made no apparent difference to the course of hydrolysis. As a general approximation it would appear that treatment for 10 min. by method (*b*) is equivalent to 24 hr. by method (*a*).

Ionophoresis of peptides

Paper ionophoresis was normally carried out by the method of Michl (1951) (Ryle, Sanger, Smith & Kitai, 1955). Except where otherwise stated, the separations were done on Whatman no. 52 filter paper with a voltage gradient of 40 v/cm. The most useful buffer for the fractionation of the phosphoserine peptides was pyridine–acetate, pH 3·5 (pyridine–acetic acid–water; 1:10:189, by vol.). The relative rates of the different peptides are very sensitive to slight changes in pH and it will be noted that the fractionations obtained in this work are rather different from those reported previously (Hartley *et al.* 1959). Although the buffer used was the same it seems that the actual pH on the paper will depend on the amounts of pyridine and acetic acid dissolved in the toluene which will be in equilibrium with the buffer on the paper. In the present experiments the toluene contained 0·3% (v/v) of acetic acid. The buffer at pH 6·5 contained pyridine–acetic acid–water (10:0·4:90, by vol.) and the toluene used in the tank with it contained 8% (v/v) of pyridine.

Each peptide fraction to be analysed, which should not contain more than about 0·1 mg. of material, was applied as a thin line over 2 cm. to the dry paper. Samples to be compared, as for instance in comparing the hydrolysates of the di*iso*propoxyphosphinyl derivatives (e.g. Fig. 11), or where peptides were run with a control mixture for identification, were applied with no gap between them but with slight overlapping (about 0·2 cm.). In this way a rigorous test for the identity of any two bands is obtained, since identical substances should give a continuous band between the two samples. After application of the samples the rest of the paper was wetted with buffer in such a way that it flowed evenly in towards the origin from each side, thus sharpening the bands of the applied samples.

In order to obtain satisfactory radioautographs each sample applied should contain at least 1 μmc of ^{32}P. To prepare the pH:mobility curves (Fig. 10) ionophoresis was carried out between cooled plates (Gross, 1955), since there was less flow of buffer into the paper from the electrode vessels than in the toluene-cooled system.

Two-dimensional ionophoresis. In order to prepare comparative two-dimensional ionophoresis patterns as shown in Fig. 12, the following technique was found useful. The samples to be compared were applied side by side and run in one dimension; the paper was then dried and a radioautograph was prepared (Fig. 11). A strip 0·5 cm. wide was then cut from the part of the paper that was to be run in a second dimension and the peptides were transferred to another sheet of paper by blotting. To do this a pad of clean filter paper slightly larger than the strip was wetted with water and blotted so that there was no excess of moisture. The strip was then wetted by pressing on to the pad with a thin glass plate. It was then transferred and pressed firmly on to the origin line of a clean sheet of paper. The process was repeated once or twice. This method transferred about 50% of the material to the new paper, which was then subjected to ionophoresis in a different buffer. An alternative method of doing two-dimensional ionophoresis which gives rather compact spots is to use cellulose acetate membrane (Kohn, 1957) as support during the first run. The cellulose acetate strip was wetted with buffer (pH 3·5) before application of the sample. Otherwise it was run under toluene in the same manner as the paper strips and a radioautograph prepared. The bands were sharp but usually rather uneven (see Fig. 5*a*). There was considerably more electroendosmosis than on paper and the relative rates of some of the peptides were slightly different although the buffer was the same. A strip about 0·5 cm. wide was cut from the cellulose acetate at a place where the bands were running evenly. A sheet of Whatman

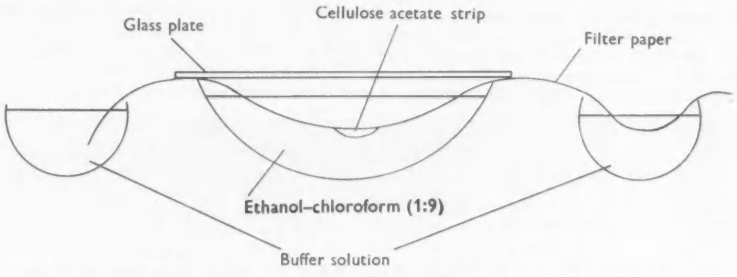


Fig. 1. Method of transferring material from a cellulose acetate strip to filter paper for two-dimensional ionophoresis.

no. 52 paper was wetted with buffer at pH 6·5 and the cellulose acetate strip pressed on to the origin line so that it was wetted with the buffer. While in this position it was wetted with a small amount of ethanol–chloroform (1:9) from a pipette. This was allowed to evaporate, thus sticking the cellulose acetate to the paper. The whole sheet was then inverted and the part around the origin line was soaked in ethanol–chloroform (1:9) as shown in Fig. 1. The paper was kept wet by immersion of its ends in troughs containing the buffer solution. The cellulose acetate dissolved in the ethanol–chloroform, leaving the radioactive spots on the paper. After soaking for about 2 hr. the paper was transferred to the ionophoresis tank for running at pH 6·5. Fig. 5*b* is a radioautograph of a paper on which a two-dimensional ionophoresis had been carried out in this way.

Isolation of peptides

In most experiments in which the peptides were studied by radioautography only small amounts of the hydrolysates were used and no preliminary separation of the phosphoserine peptides was necessary. However, in order to obtain sufficient amounts of peptides to determine their amino acid content and end groups, two experiments were done on a somewhat larger scale. Di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin (100–200 mg.) was hydrolysed with acid (method *a* for 2 days or method *b* for 20 min.). The HCl was removed *in vacuo* with a rotary evaporator and the residue was dissolved in 2 ml. of water and applied to a column (2 cm. diam.) of Dowex-50 (10 g., in the acid form). The column was developed with water. The material coming through with the solvent front was collected and contained over 90% of the added radioactivity. This treatment, which removes the majority of the peptides not containing serine phosphate (Flavin, 1954), was necessary to avoid overloading during ionophoresis. After repeated evaporation *in vacuo* the peptide mixture was applied as a 35 cm. band 10 cm. from the cathode end of a sheet of Whatman no. 52 paper. Ionophoresis was carried out at pH 3·5 at 40 v/cm. for 2·5 hr. A radioautograph was prepared, a part of which is shown in Fig. 4. The bands were cut out and eluted with water. Each peptide was purified by ionophoresis at pH 6·5. Samples were taken for hydrolysis and for end-group determination by the 2:4-dinitrophenyl technique. Where possible 0·1 μmole of peptide was used for hydrolysis. The amounts of the various peptides were estimated from the radioactivity.

The amino acids in the hydrolysates were identified by ionophoresis in 2·5% (v/v) formic acid for 25 min. at 80 v/cm. This system gives very sharp bands and clearly separates all the amino acids expected in these peptides (Ser, Asp, Gly, Glu, Ala). *N*-Terminal residues were determined by the 2:4-dinitrophenyl technique, dinitrophenyl-amino acids being identified by paper chromatography on *tert.*-amyl alcohol–phthalate buffer, pH 5·0 (Blackburn & Lowther, 1951).

Edman degradation

In the Edman degradation it is usual to identify the *N*-terminal residue as the phenylthiohydantoin or as the amino acid missing from a hydrolysate of the phenylthiocarbamyl peptide. We have used a somewhat different approach and have identified the radioactive product produced. This gives considerable information about the relationships between the different peptides. Various modifications of the original Edman method have been employed. Most of the results reported here were obtained by the procedure described below, which is a combination of various published methods (Edman, 1950; Fraenkel-Conrat & Harris, 1954).

The radioactive peptide (containing at least 1 μmc of ^{32}P) was taken to dryness in a small test tube and dissolved in 0·1 ml. of water. Phenyl*iso*thiocyanate–pyridine (0·1 ml.; 1:9, v/v) was added and the mixture shaken at room temperature for 3–5 hr. Benzene (2 ml.) and a few drops of water were added, and the mixture was shaken and the benzene layer removed. The extraction was repeated and the aqueous residue taken to dryness over H_2SO_4 in a desiccator. After addition of water and evaporation two or three times, 0·2 ml. of 3 N-HCl was added and the mixture allowed to stand for 5 hr. at room temperature. The HCl was removed *in vacuo* over NaOH and the residue subjected to ionophoresis at pH 3·5. A partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin was run in parallel as a control in order to be able to identify the product.

We have also used the method of Sjöquist (1957) on some of the peptides. Little difference was noticed between the methods, but it was observed that some peptides gave much clearer results than others. Those containing *iso*propyl groups appeared to react better than those with unesterified phosphate. When peptides containing *N*-terminal serine phosphate (e.g. peptide 10 and some peptides from ovalbumin; F. Sanger, unpublished work) were subjected to this procedure, the only product present in significant amounts migrated at the same rate as inorganic phosphate. It is unlikely that the phenylthiohydantoin of serine phosphate, which is the expected product, would move so fast and it appears that the compound is unstable and breaks down to give phosphate. Similarly, peptide 11 (SerIP.Gly) breaks down to give *iso*propyl phosphate (band 2).

Radioautographs

The filter paper to be radioautographed was stuck with cellulose self-adhesive tape into a cardboard filing folder, one side of which was covered with lead sheeting (0·25 mm. thick). One or two sheets of Ilford Industrial G X-ray film were then stuck in the folder, which was kept in a light-proof box until ready for developing. Where individual bands from a sample put on over 2 cm. paper contained 1 μmc of ^{32}P the film could be developed after about 24 hr. If the bands contained 0·1 μmc about 10–15 days were required.

In order to mark the radioautographs and to be able to align them with the filter papers, the papers were marked with a radioactive pencil: commercial propelling pencil leads were immersed overnight in radioactive residues (^{14}C or ^{35}S) of high specific activity and then dried in an oven at 105°. The leads were then used in a propelling pencil.

*Time course of hydrolysis of di*iso*propoxy[^{32}P]phosphinyl enzymes*

The di*iso*propoxy[^{32}P]phosphinyl enzyme was dissolved in HCl and samples were measured into small test tubes, the size of the sample being adjusted so that the amounts put on the ionophoresis paper would be constant (about 1 μmc of ^{32}P/cm.). In most experiments each sample contained about 2 μmc and was put on the ionophoresis paper

over a 2 cm. width; however, in the experiment illustrated in Fig. 2 the size of the samples varied. Each sample was incubated at 37° or 100° for the appropriate period and after removal of the HCl *in vacuo* they were subjected to ionophoresis at pH 3·5, being put on side by side in the appropriate order (see Figs. 2 and 3).

RESULTS

Most of the results reported in this section were obtained with chymotrypsin or trypsin. However, they apply equally well to elastase since no major differences could be detected in the radioactive peptides obtained from the di*iso*propoxy[^{32}P]-phosphinyl derivatives of the three enzymes (see below).

Course of hydrolysis of diisopropoxy[^{32}P]phosphinyl enzymes

Fig. 2 shows the course of hydrolysis of di*iso*-propoxy[^{32}P]phosphinyl-trypsin by method (*b*). It can be seen that the main stable derivatives are in bands 1, 4 and 10. Bands, 3, 6, 7 and 9B are also relatively stable but are present in smaller amounts. It seemed that a period of hydrolysis of 20–30 min. gave a suitable mixture of peptides, and most of the work was carried out under these conditions. In some experiments method (*a*) was used and it was found that a suitable time of hydrolysis was 2–3 days. There were no apparent qualitative differences between the two methods of hydrolysis, so that to save time method (*b*) was normally used.

In an attempt to identify larger peptides, hydrolysates prepared by method (*c*) were also studied. The course of this reaction is shown in Fig. 3 and appears to be somewhat different from that with methods (*a*) and (*b*). A 3-day period of hydrolysis gave relatively high concentrations of a number of bands, such as 7B, 9A, 11, 14, 15 and 18, which were absent or present only in small amounts in the other more complete hydrolysates, and this time of hydrolysis was then used to study these slower-moving peptides.

Identification of radioactive bands

Fig. 4 shows a radioautograph of a typical ionophoresis experiment of a partial acid hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. On running again at pH 6·5, most of the bands were essentially pure though there were usually smaller amounts of other contaminants. Fig. 5 shows a two-dimensional ionophoresis run first on cellulose acetate at pH 3·5 and then on paper at pH 6·5, as described in the Experimental section, and the numbering of the various peptide spots is

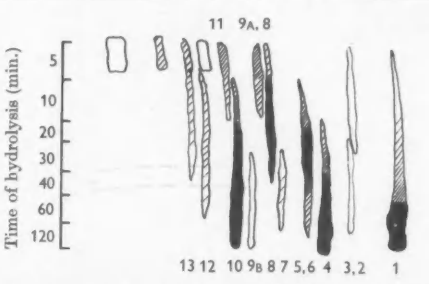


Fig. 2. Time course of hydrolysis of di*iso*propoxy[^{32}P]-phosphinyl-trypsin by method (*b*). Samples of di*iso*-propoxy[^{32}P]phosphinyl-trypsin were hydrolysed for different periods and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr.

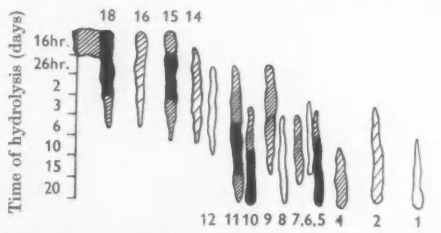


Fig. 3. Time course of hydrolysis of di*iso*propoxy[^{32}P]-phosphinyl-trypsin by method (*c*). Samples were hydrolysed for different periods and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr.

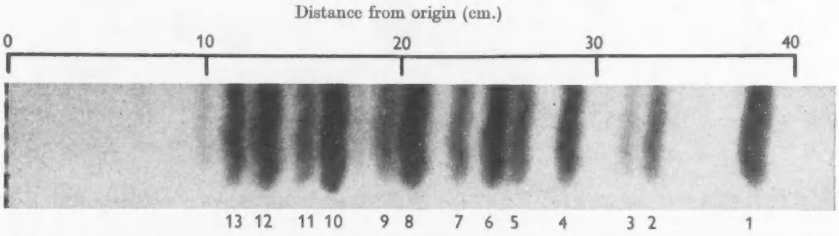


Fig. 4. Radioautograph of ionophoretic fractionation of partial acid hydrolysate (method *b*, 20 min.) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. Ionophoresis at pH 3·5, 40 v/cm., 2·5 hr.

shown in Fig. 6. This is a diagram of a two-dimensional ionophoresis pattern carried out entirely on filter paper and is thus slightly different from Fig. 5. It incorporates all the main peptides studied in this work, including those in the hydrolysate obtained by method (*c*) (3 days).

The yields of peptides from a hydrolysate (method *a*, 2 days) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin are given in Table 1. The total recovery of ^{32}P is low (62 %) so that there have been considerable losses, probably mainly during elution of the peptides from the filter paper and running again at pH 6·5.

The results of the large-scale experiments, in which some of the peptides were hydrolysed to identify the amino acids present, and in which the end groups of some peptides were determined, are shown in Table 2. This method of analysis clearly requires that the peptides should be free of any contaminating non-radioactive peptides. Most of these should have been removed on the Dowex-50 column and should not move as acids at pH 3·5.

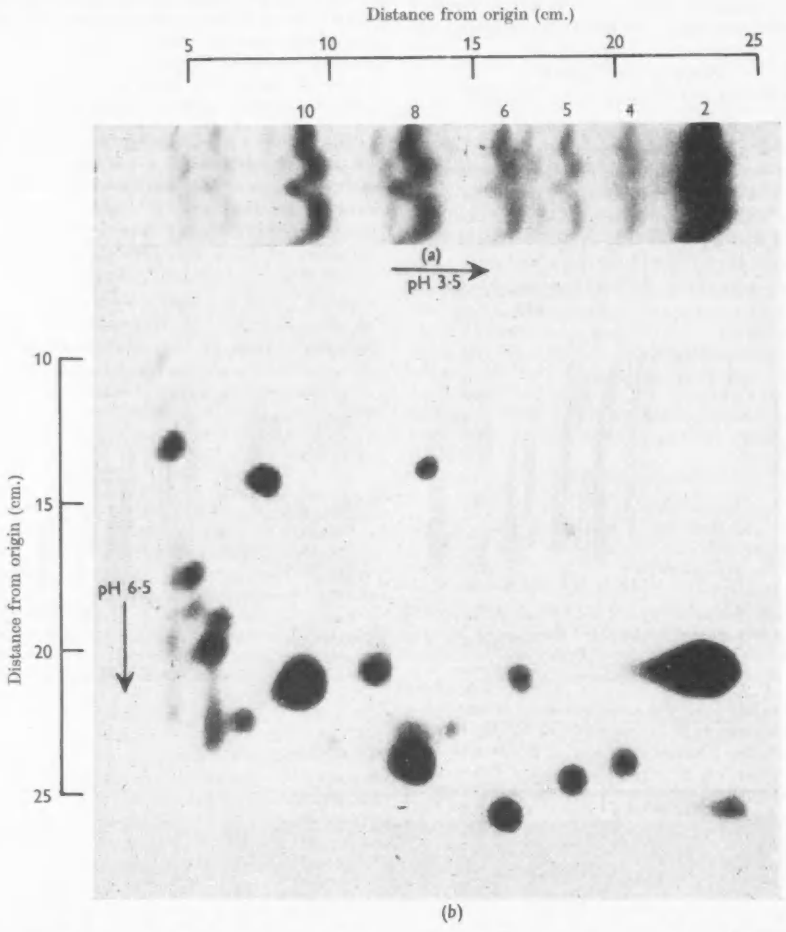


Fig. 5. Ionophoresis of acid hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-trypsin. (*a*) First dimension on cellulose acetate, pH 3·5, 40 v/cm., 2·5 hr. (*b*) First dimension as in (*a*), second dimension on filter paper, pH 6·5, 40 v/cm., 1·75 hr. The strong spot 2 is due to contamination of the di*iso*propoxy[^{32}P]-phosphinyl-trypsin with DF^{32}P, caused by inadequate dialysis of the preparation.

However, the presence of glutamic acid in the hydrolysate of band 5A before purification at pH 2·1 indicates that some contamination may take place. It was at first considered that peptide 5A was [Asp, SerP, Gly, Glu]. However, this could not be reconciled with the partial-hydrolysis results, so that further purification was attempted.

Each band was partially hydrolysed (usually by method *b*, 30 min.) and subjected to ionophoresis at pH 3·5. A sample of a hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin was run adjacent to each peptide hydrolysate as a control in order to be able to identify the various degradation products. The results are given in Table 3. The yields given were estimated by eye from the darkening of the radioautographs and indicate the relative amount of each product obtained from a given quantity of each peptide band. The letters 'tr' indicate the presence of a small but significant amount of the product. A small amount of free phosphate (band 1) was obtained from each band but this is not included in the Table 3.

Since the course of hydrolysis by method (*c*) is somewhat different, the main products obtained by this method were also subjected to a further hydrolysis for 3 days to show their inter-relationships. These results are given in Table 4.

Samples of some of the peptides were subjected to the Edman degradation procedure and the product was identified by ionophoresis at pH 3·5. The results were not always entirely clear-cut. Unchanged material was frequently present and varying amounts of free phosphate. The results of experiments where the main product of the degradation was clear are given in Table 3.

Two of the bands could be identified directly by comparison of their rates with marker compounds of known structure. These were band 1, which was identified as free phosphate, and band 4, which was serine phosphate. Band 2 is present together with band 1 in a mild acid hydrolysate (e.g. method *c*, 1–3 days) of DF^{32}P. On hydrolysis it gives rise to band 1 only and appears to be mono*iso*propyl phosphate.

The only peptide band that was readily identifiable was band 10, which behaved essentially as a dipeptide on partial acid hydrolysis, giving only serine phosphate in significant amounts. It was

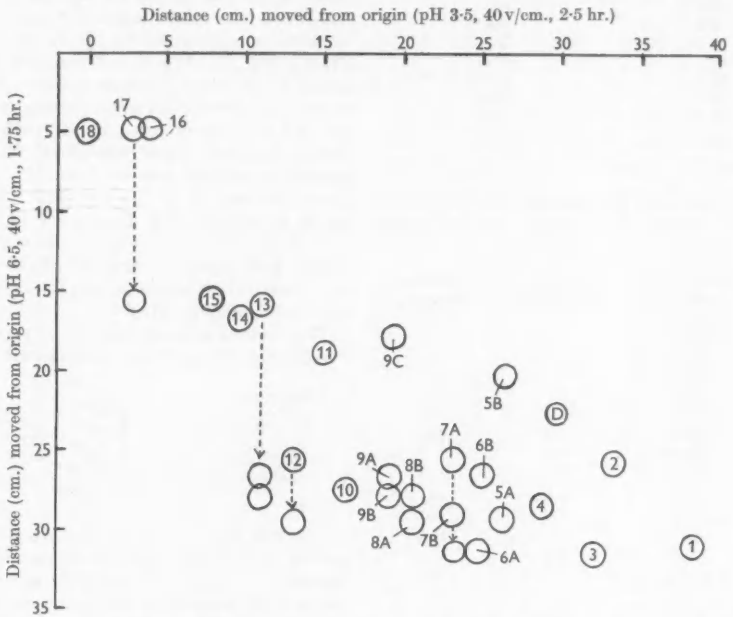


Fig. 6. Diagram showing the migration rates at pH 3·5 and pH 6·5 of radioactive compounds obtained from partial acid hydrolysates of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. Ionophoresis on Whatman no. 52 filter paper. The materials were applied near to the cathode end of the paper. There was considerable flow of buffer into the paper from both ends during the ionophoresis. Spot D shows the position occupied by di*iso*propyl phosphate. The broken arrows indicate the changes that take place during running at pH 6·5.

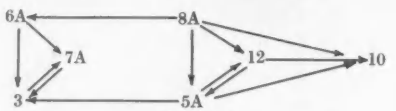
the most stable peptide (Fig. 2) and gave serine and glycine on complete hydrolysis. It was clearly SerP.Gly.

Although the main product of partial hydrolysis of SerP.Gly was serine phosphate, two other bands were given in small amounts. They moved at the rates of bands 9B and 10*x* (Fig. 7). Peptide 9B appears during the later stages of hydrolysis by method (*b*) and is very stable. On partial hydrolysis it gives band 4 and a trace of band 10. It is formed in larger amounts if peptide 10 is hydrolysed in more dilute acid (e.g. 3N-HCl, 100°; Fig. 7, no. 4). On Edman degradation it gave serine phosphate. It seems probable that it is Gly.SerP formed by inversion of SerP.Gly via the intermediate anhydride. This reaction has been shown to occur during hydrolysis in 2N-HCl (Schaffer, Harshman & Engle, 1955) and also appears to occur in 5·7N-HCl at 100°, though it was probably not significant when method (*a*) was used.

The nature of band 10*x* is unknown. Its yield varies considerably and is greatly increased if the partial hydrolysis is carried out in the presence of a small amount of glucose (Fig. 7, no. 5) or of filter paper. It would thus appear to be an artifact formed from SerP.Gly due to the presence of impurities (probably carbohydrate) extracted from the filter paper. Its slow rate of migration at pH 3·5 suggests decarboxylation or else that one of the acidic groups is substituted.

The results with the other main peptide bands could not readily be explained in any simple way. A number of bands were interconvertible. For instance, peptide 5A on partial hydrolysis gave 12 and 12 gave 5A. When certain bands were run again as controls at pH 3·5 they were found to be converted into other bands and these conversions could be brought about by incubation overnight at pH 6·5 (Fig. 8). Thus 12 was completely converted into 5A, 13 which appears to be a mixture was converted into two peptides moving at the rates of 6B and 8B respectively. Band 7 was partly converted into band 3 and was partly unchanged. It seemed to contain two compounds: 7A, which is converted into 3 at pH 6·5, and 7B, which is stable at pH 6·5. The decomposition of bands 12 and 13 can be seen in Fig. 5 by the streaks originating from spots 12 and 13. Evidently in this experiment the conversion was taking place during the ionophoresis at pH 6·5.

The various interconversions of peptides 3, 6A, 7A, 5A, 8A, 12 and 10 are summarized in Scheme 2.



Scheme 2

The fact that 3, 6A and 7A are related and on partial hydrolysis give only serine phosphate and one another suggested that they were all different forms of the same dipeptide, presumably Asp.SerP. Peptides 5A, 8A and 12 were also similarly related and broke down to give 10 (SerP.Gly) and different forms of Asp.SerP. They thus appeared to be the corresponding three forms of the tripeptide Asp.SerP.Gly.

Table 1. *Yield of peptides from hydrolysate (method a, 2 days) of di*isopropoxy[^{32}P]*phosphinyl-chymotrypsin*

Yield is expressed as ^{32}P recovered in peptide band as percentage of ^{32}P of original di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin.

Band	Yield
1	5·0
2	1·5
3	2·4
4	3·0
5A	11·0
5B	0·2
6A	1·5
6B	1·0
7 (A+B)	5·0
8A	9·0
8B	1·4
9 (A+B+C)	3·0
10	10·7
11	1·6
12	2·3
13 (A+B)	3·2
Total yield	61·8

Table 2. *Amino acid composition and N-terminal residues of peptides from di*isopropoxy[^{32}P]*phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Amino acids present	Approx. strength	*N*-Terminal residue
1	None	—	—
2	None	—	—
4	Ser	× × ×	—
	SerP	×	—
	Glu	tr	—
5A*	Ser	× ×	Asp
	Gly	× ×	—
	Asp	× ×	—
6A	Ser	× ×	Asp
	Asp	× ×	—
8A	Ser	× ×	Asp
	Gly	× ×	—
	Asp	× ×	—
	Glu	tr	—
10A	Ser	× ×	—
	Gly	× ×	—

* Band 5A, after purification by ionophoresis at pH 3·5 and 6·5, contained glutamic acid (× × ×). This was not present after further ionophoresis in 2·5% (v/v) formic acid (pH 2·1).

Table 3. *Partial hydrolysis and Edman degradations of serine phosphate peptides from di*isopropoxy[^{32}P]*phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Peptide bands given after hydrolysis (method *b*, 30 min.)									Main product of Edman degradation	Probable structure
	3	4	5	6	7	8	10	12	Other bands		
3	× ×	× ×	—	—	×	—	—	—	—	—	β-Asp.SerP
4	—	×	—	—	—	—	—	—	—	1	SerP
5A	× ×	× ×	× × ×	—	×	—	× × ×	×	—	—	β-Asp.SerP.Gly
5B	—	×	—	—	—	—	—	—	—	—	SerIP
6A	tr	× ×	—	× ×	tr	—	—	—	—	4	α-Asp.SerP
6B	×	× ×	×	—	tr	—	× ×	tr	—	—	β-Asp.SerIP.Gly
7A*	× ×	× × ×	—	—	×	—	—	—	—	—	αβ-Asp.SerP
7B*	tr	× × ×	—	× ×	tr	—	—	—	—	5B	α-Asp.SerIP
8A	tr	× ×	tr	× ×	tr	× × ×	× × ×	tr	—	10	α-Asp.SerP.Gly
8B	×	× ×	× ×	—	tr	×	× × ×	×	13 (×)	5†	Gly.β-Asp.SerP.Gly?
9A	tr	× × ×	tr	× ×	tr	× ×	× × ×	tr	—	11	α-Asp.SerIP.Gly
9B	—	× ×	—	—	—	—	tr	—	9 (× ×)	4	Gly.SerP
9C	×	× × ×	—	—	× ×	—	—	—	9 (× ×)	—	?
10	—	×	—	—	—	—	× × ×	—	9B (tr), 10*x* (tr)	1	SerP.Gly
11	—	×	—	—	—	—	× × ×	—	9B (tr), 10*x* (tr)	2	SerIP.Gly
12	× ×	× ×	× × ×	—	×	tr	× × ×	×	—	—	αβ-Asp.SerP.Gly
13A‡	×	× ×	×	—	×	—	× ×	tr	13 (tr)	—	αβ-Asp.SerIP.Gly
13B‡	×	× ×	×	—	×	tr	× ×	×	13 (×)	—	Gly.αβ-Asp.SerP.Gly?
14	tr	× × ×	—	× ×	tr	—	—	—	—	16	α-Asp.SerDIP
15	tr	× ×	tr	×	tr	×	× ×	tr	9A (tr)	18	α-Asp.SerDIP.Gly
16	—	×	—	—	—	—	—	—	—	—	SerDIP
17	tr	× ×	tr	×	×	tr	× ×	tr	—	—	αβ-Asp.SerDIP.Gly?
18	—	×	—	—	—	—	× × ×	—	2 (× ×)	—	SerDIP.Gly

* Band 7 was incubated at pH 6·5 overnight and refractionated at pH 3·5. 7A was the fast-moving band (3), 7B the slower-moving one. 7B was obtained in higher yield directly from the 3-day hydrolysate by method (*c*).

† Result of a single experiment. There was a relatively large amount of unchanged peptide present.

‡ Band 13 was incubated at pH 6·5 overnight and refractionated at pH 3·5. 13A moved at the rate of band 6, 13B at the rate of band 8.

Table 4. *Partial hydrolysis (method c) of peptides from diisopropoxy[^{32}P]phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Peptide bands given after partial hydrolysis (method *c*, 3 days)									
	5*	7B	9A	10	11	14	15	17	18	Other bands
7B	tr	× ×	—	—	—	—	—	—	—	—
9A	tr	×	× ×	—	×	—	—	—	—	8 (tr), 13 (tr)
11	tr	—	—	tr	× ×	—	—	—	—	2 (tr)
14	×	×	—	—	—	× ×	—	—	—	—
15	tr	×	×	tr	×	×	× ×	tr	×	2 (tr)
18	—	—	—	tr	× ×	—	—	—	×	2 (× ×)

* Bands 5 and 6 were not well resolved. It is probable that the band in this position is 5B though it could in some cases have been 5A, 6A or 6B.

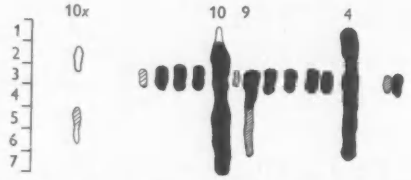


Fig. 7. Breakdown of peptide 10 under various conditions of hydrolysis. Samples of peptide 10 (containing ^{32}P, 5 μmc) hydrolysed as below and subjected to ionophoresis at pH 3·5, 40 v/cm., 2·5 hr. 1, Method (*b*), 3 hr.; 2, method (*a*), 3 days; 3, control hydrolysate (method *b*, 30 min.) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin; 4, 3 N-HCl, 100°, 6 hr.; 5, as for 6 with a trace of glucose added; 6, method (*b*), 35 min.; 7, untreated.

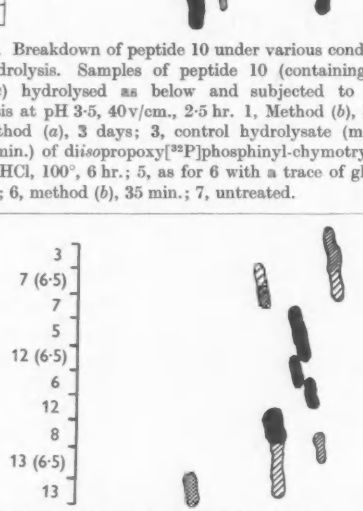


Fig. 8. Decomposition of peptides 7, 12, 13 after incubation at pH 6·5, for 16 hr. Other markers were eluted from papers on which they had been fractionated by ionophoresis, dried down and run again (1 μmc per sample). Ionophoresis: pH 3·5, 40 v/cm., 2 hr.

These results are best explained by assuming that 6A and 8A are normal α-aspartyl peptides, 7A and 12 the relatively unstable ring forms (αβ-aspartyl derivatives) and 3 and 5A the β-aspartyl peptides. The assignment of the β form to 3 and 5A was at first based largely on their greater ionophoretic rate at pH 3·5. Since the aspartic acid is *N*-terminal in both peptides, the aspartic acid carboxyl group in the β form will be adjacent to an α-amino group and will have a lower p*K* than

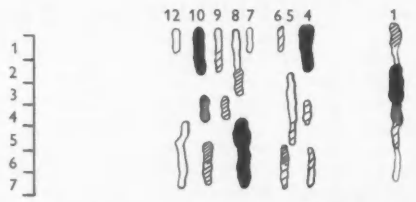


Fig. 9. Breakdown of peptide 8 under various conditions. Samples of peptide 8 (containing ^{32}P, 1 μmc) were treated as below and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr. 1, Method (*b*), 55 min.; 2, 2 N-HCl, 100°, 2 hr.; 3, water, 100°, 6 hr.; 4, 20% (v/v) formic acid, 100, 4 hr.; 5, thionyl chloride, 18°, 18 hr.; 6, 6 N-HCl, 120°, 3 min.; 7, method (*b*), 25 min.

the normal β-carboxyl group and hence move faster at pH 3·5. Peptide 3 is formed during the later stages of hydrolysis and seems to be relatively stable. John & Young (1954) showed that α-Asp.Val can be converted into β-Asp.Val by heating in water at 100° for 6 hr. Under these conditions considerable conversion of 8A into 5A and of 6A into 3 occurred (see Fig. 9). The various results given in Tables 2–4 are in agreement with the above interpretation and can thus be regarded as confirmation of it.

Probably the best method of characterizing the various aspartyl derivatives would be by their titration curves (see Swallow & Abraham, 1958). Since we had insufficient material for this we attempted to determine the p*K* values of the various groups by measuring the ionophoretic mobility of the peptides at different pH values. If the mobility is plotted against pH, the resulting curve should be analogous to a titration curve. In order to compensate for electroendosmosis, for the flow of buffer into the paper from the electrode vessels and for variations in conditions of running, the results were expressed relative to a marker of known titration curve. For this purpose serine phosphate was used and the radioactive material (from band 4) was run together with the peptides at each pH value. A glucose marker was also used

to determine the displacement of the origin. A titration curve of serine phosphate was then prepared in the normal way, and the net charge was calculated for the different pH values. From this the theoretical mobility of serine phosphate was determined, assuming a value at pH 5·1 of 13 cm. This was the experimental value found after ionophoresis for 1·5 hr. at 30 v/cm. The relative mobilities for the peptides were calculated as

$$\frac{\text{Distance of peptide from glucose marker}}{\text{Distance of serine phosphate from glucose marker}} \times \text{theoretical mobility for serine phosphate.}$$

The results are, then, the distance travelled by the peptide in 1·5 hr. at 30 v/cm. under the experimental conditions (heating etc.) that prevailed during the experiment at pH 5·1. Since the absolute mobilities could not be determined it was considered preferable to express the results in these arbitrary units.

Some of the curves so obtained are shown in Fig. 10. Although they cannot be regarded as very accurate owing to the limitations of the method, they do give an indication of the pK values of the various groups involved and clearly show that one of the carboxyl groups and the amino groups in 3 and 5A are considerably stronger than the corresponding groups in 6A and 8A. Unfortunately 7A and 12 were too unstable at most pH values to determine their mobilities. One of the pK values of the phosphate group is about 5·5–6·0 (Fölsch & Österberg, 1959), and this is absent from peptide 11, thus confirming that it is a mono*iso*propyl derivative (see below). The pK values of the amino groups decrease with increased peptide size (4 > 6A > 8A), as would be expected.

Fig. 9 shows the results of various treatments on peptide 8 (α-Asp.SerP.Gly). The main products of hydrolysis are normally 4, 6, 10 and 12 (e.g. in sample 7), the relative amounts of each depending on the time of hydrolysis. Treatment with 20% (v/v) formic acid (sample 4) leads almost exclusively to peptide 10 (SerP.Gly) and its inversion product 9B. This is in agreement with the results of Partridge & Davis (1950), who showed a preferential splitting of bonds involving aspartyl residues in weak acid.

The action of thionyl chloride was studied to see if it converted an α-aspartyl peptide (e.g. peptide 8) into the αβ form (e.g. peptide 12). This does take place to a small extent, though most of 8 was unchanged under the conditions used. A more appreciable conversion of peptide 6 into 7 was brought about by treatment with thionyl chloride at 100° for 15 min. This can be regarded as further support for the conclusion that 7A and 12 are αβ forms.

A number of peptides, especially in the hydrolysate prepared by method (*c*) for 3 days, contain the phosphate as the mono*iso*propyl or di*iso*propyl derivatives. These can be recognized by their relative rates of migration at pH 3·5 and 6·5, since the second ionization constant (pK 5·5–6·0) of the phosphate is blocked in them. Also, on treatment with alkali (4% triethylamine, 30 min., 105°) the mono*iso*propyl derivatives gave mono*iso*propyl phosphate (band 2), which could be identified. The following bands were shown in this way to be mono*iso*propyl derivatives: 5B, 7B, 9A, 11. Their probable structure was deduced from the products of their hydrolysis and is shown in Table 3. Peptides 14, 15, 16, 17 and 18 appeared from their behaviour on degradation to be di*iso*propyl derivatives. When they were treated with triethylamine and the product was subjected to iono-

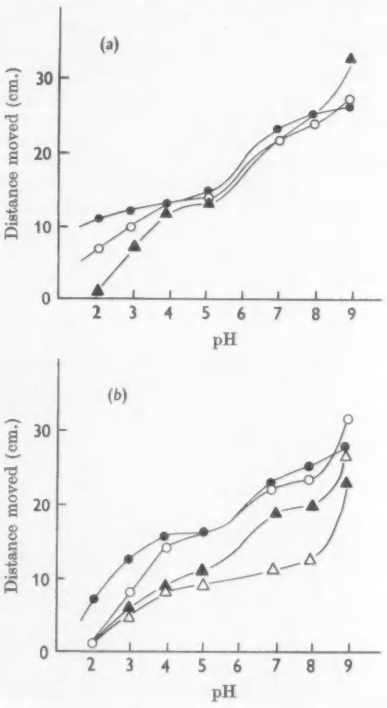


Fig. 10. Mobility–pH curves for ^{32}P-containing bands from partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. The curves are plotted relative to the theoretical distance of migration of serine phosphate at 30 v/cm. for 1·5 hr. (see text). (*a*) ●, Serine phosphate (theoretical curve); ○, band 5A; ▲, band 8A. (*b*) ●, Band 3; ○, band 6A; ▲, band 10A; △, band 11.

phoresis at pH 3·5 under toluene the radioactivity was almost completely lost. This also occurred when DF^{32}P was treated in the same way. If, however, the ionophoresis was done between cooled plates a band was obtained from the alkaline degradation of DF^{32}P and of peptide 15 which moved slightly faster than serine phosphate. It is presumably di*iso*propyl phosphate. It appears that it is extracted into toluene, possibly in the form of its triethylamine salt.

Schaffer *et al.* (1957) identified the peptide Gly.Asp.SerP.Gly in hydrolysates of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin (method *a*, 3 days). It seems most likely that band 8B has this structure, although it was present in rather small amounts and was not completely characterized. It is clearly in the β form and 13B is the corresponding $\alpha\beta$ form. Schaffer & Lang (1959) have reported the identification of a labelled peptide having the amino acids [Ser.Gly.Glu.Ala] (see also Turba & Gundlach, 1955). We have been unable to detect any peptide that could have this structure. There are a number of weak bands that have not been thoroughly characterized. The most likely structure for 9C is $\alpha\beta$-Asp.SerIP. On running again at pH 3·5 and on incubation with alkali it gives rise to a peptide moving between bands 3 and 4, which could be β-Asp.SerIP.

When band 10 (SerP.Gly) was repurified by ionophoresis at pH 6·5 a small amount of a rapidly moving band was always present. This proved to be free phosphate. Similarly, when bands 11 (SerIP.Gly) and 18 (SerDIP.Gly) were purified at pH 6·5, faster-moving bands were present which could have been *iso*propyl phosphate and di*iso*propyl phosphate respectively. It would thus appear that these peptides are somewhat unstable to the conditions employed. There were several other bands present in small amounts whose structures could not be determined. The results with these are not recorded here.

Comparison of ^{32}P*-containing peptides from the di-isopropoxy*[^{32}P]*phosphinyl derivatives of chymotrypsin, trypsin and elastase*

Samples of the di*iso*propoxy[^{32}P]phosphinyl derivatives of trypsin, chymotrypsin and elastase were hydrolysed for 20 min. by method (*b*) and subjected to ionophoresis in parallel together at pH 3·5. Fig. 11 is a radioautograph of the paper which shows no difference between the three enzymes. By the use of the blotting method two-dimensional ionophoresis patterns were prepared from the above-mentioned parallel one-dimensional ones and these are shown in Fig. 12. A shorter-time hydrolysis (10 min.) of di*iso*propoxy[^{32}P]phosphinyl-elastase is also shown. These results reveal no differences in the ^{32}P-containing peptides from the three enzymes.

DISCUSSION

The above-described results show that the complexity of the hydrolysates of the di*iso*propoxy-[^{32}P]phosphinyl-enzymes is largely due to two factors: the incomplete hydrolysis of the *iso*propyl

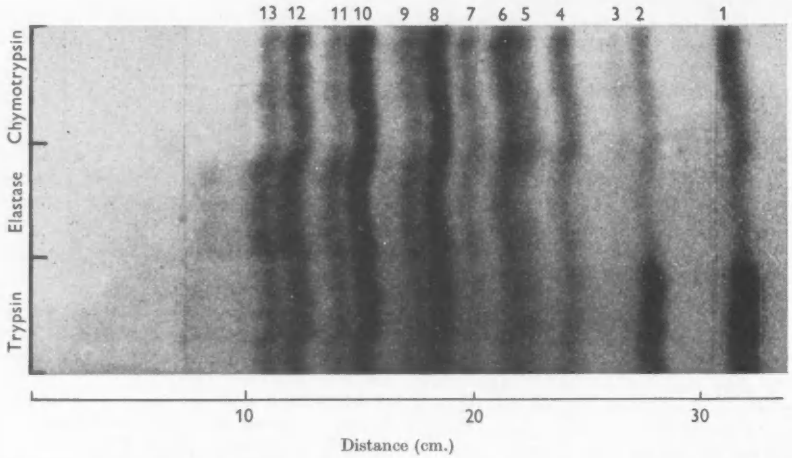


Fig. 11. Ionophoresis of partial hydrolysates (method *b*, 20 min.) of the di*iso*propoxy[^{32}P]phosphinyl derivatives of chymotrypsin, elastase and trypsin, pH 3·5, 40 v/cm., 2 hr. The strong bands 1 and 2 from the trypsin derivative are derived from DF^{32}P, which contaminated the preparation.

ester groups and the aspartyl-interconversion reaction. It seems likely that this interconversion is a general reaction and is to be expected in experiments where proteins or peptides containing aspartic acid are subjected to hydrolysis with acid. It probably occurred during the hydrolysis of fraction A of oxidized insulin with acid (Sanger & Thompson, 1953), since three peptides (A1a9, A1a10, A1a14) with the apparent structure Asp.Tyr were detected. They were explained as being due to the presence of an amide group and to TyrX (chlorotyrosine; Thompson, 1954). It is unlikely that amide groups would in fact be stable under the conditions of hydrolysis and a more likely explanation is that peptide A1a14, which gave a yellow colour with ninhydrin, was the β-Asp.Tyr. John & Young (1954) report that β-aspartyl peptides give a yellow ninhydrin colour.

Although Schaffer *et al.* (1957) did not detect the interconversion in their experiments, which were done under the same conditions as we have used, it is probable that it occurred and that their peptide 2 was the same as our peptide 5A (β-Asp.SerP.Gly), whereas peptide 3 was our 8A. They found that although both reacted with phenyl *iso*thiocyanate in the Edman procedure, the

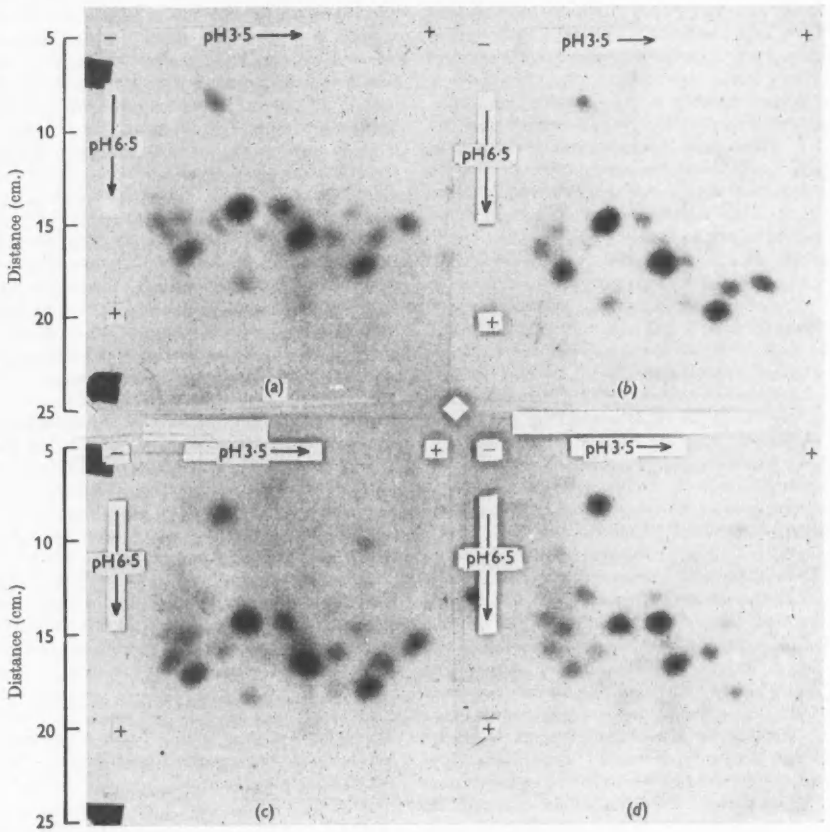


Fig. 12. Comparative two-dimensional ionophoresis patterns of partial hydrolysates (method *b*) of the di*iso*propoxy[^{32}P]phosphinyl derivatives of trypsin, chymotrypsin and elastase. First dimension, pH 3·5, as in Fig. 11; second dimension, pH 6·5, 40 v/cm., 1 hr. Transfer to second dimension was by the blotting technique (see text). (*a*) Di*iso*propoxyphosphinyl-trypsin, 20 min. hydrolysate. (*b*) Di*iso*propoxyphosphinyl-chymotrypsin, 20 min. hydrolysate. (*c*) Di*iso*propoxyphosphinyl-elastase, 20 min. hydrolysate. (*d*) Di*iso*propoxyphosphinyl-elastase, 10 min. hydrolysate.

phenylthiohydantoin of aspartic acid was liberated only from peptide 3 and not from peptide 2, as would be expected if the latter was a β-aspartyl peptide.

Various methods of hydrolysis were studied (e.g. Fig. 9) in an attempt to find conditions in which the interconversions of aspartic acid peptides could be avoided. No such conditions could be found but the reaction seems to be less marked in 5·7N-hydrochloric acid than in 12N-hydrochloric acid. The main difference between hydrolyses in 12N-hydrochloric acid (method *a*) and in 5·7N-hydrochloric acid (method *c*) at 37° is in the stability of the *iso*propyl groups, which are much more resistant under the latter conditions. There was no evidence of any significant amounts of peptides larger than tripeptides in the 3-days hydrolysate (method *c*). Only relatively small amounts of the β and $\alpha\beta$ forms of the aspartyl peptides were present. It also appears that the interconversion reaction is less marked when hydrolysis is carried out by method *b* than when carried out by method *a*. There was no evidence of any inversion of a dipeptide sequence with method (*c*) so that it seems that in general this would be a better method to use if side reactions are to be avoided.

From the identity of the radioautographs of the ionograms of partial hydrolysates of the di*iso*propoxy[^{32}P]phosphinyl derivatives of elastase, chymotrypsin and trypsin, it was concluded that elastase, like the other enzymes, contained the sequence Gly.Asp.SerP.Gly (Hartley *et al.* 1959). This conclusion was based on the assumption that only three peptides (Asp.SerP, SerP.Gly and Asp.SerP.Gly) would be derived from the sequence Asp.SerP.Gly. In fact six to seven identical bands were obtained from the three enzymes. Hence it was assumed that they must have more than three residues in common; Schaffer *et al.* (1957) had shown the presence of Gly.Asp.SerP.Gly in hydrolysates of the type we were using. Clearly in view of the rearrangement reactions of the aspartyl peptides, the above conclusions are no longer valid. In fact in the present work we have shown that the hydrolysates of the three di*iso*propoxy[^{32}P]phosphinyl enzymes have about 20 peptides in common but can only conclude for certain that they all have the sequence Asp.Ser.Gly. Owing to the interconversion reaction and to the presence of mono- and di-*iso*propyl esters, each aspartic acid peptide can occur in nine forms, which greatly complicates the composition of the hydrolysates. It is probable that peptide 8B is Gly.Asp.SerP.Gly, and it seems to be present in the hydrolysates of di*iso*propoxy[^{32}P]-phosphinyl-chymotrypsin, -trypsin and -elastase (Fig. 12), suggesting they have the tetrapeptide sequence in common. However, the amount of this peptide is very small and its structure uncertain so that the suggestion cannot be regarded as conclusive.

The possibility has to be considered whether the aspartyl residue in the intact proteins could be the β or $\alpha\beta$ form rather than the normal α form. This seems unlikely since the proportion of the β forms increases with time of hydrolysis and the conversion of a β form into an α form (e.g. 5A → 8A) has not been observed on partial acid hydrolysis of the isolated peptides, whereas the reverse reaction does take place (e.g. 8A → 5A). On the other hand the yields of the β and $\alpha\beta$ forms do seem to be unexpectedly high. Table 5 shows the relative yields of bands 8A (α-Asp SerP Gly), 5A (β-Asp SerP. Gly) and 12 ($\alpha\beta$-Asp SerP Gly) from a partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin and from a partial hydrolysate of peptide 8A. It can be seen that the relative yields of the β and $\alpha\beta$ forms are greater from the protein than from the isolated peptide. This can probably best be explained by assuming that the conversion is more rapid if the α-amino group of the aspartyl residue is blocked as in a larger peptide, so that most of the reaction takes place during the early stages of hydrolysis of the protein and the β forms (e.g. 3 and 5A) are probably produced largely from the β and $\alpha\beta$ forms of larger peptides rather than from the corresponding α forms (6A and 8A). In this connexion if 8B is Gly β-Asp SerP Gly and 13B its $\alpha\beta$ form, there is no evidence of the α form which suggests that the longer period of survival of the larger peptide in the acid has led to its complete conversion. Whereas it seems most likely that the natural form of the aspartyl residue is the α form, the possibility cannot be excluded that it may be partly in the β or $\alpha\beta$ form. It seems unlikely that the reaction could be involved in the enzymic activity of the proteins, since it appears to be brought about by acid treatment; however, again the possibility cannot be excluded.

It must, of course, be more than coincidence that the three proteolytic enzymes studied have the sequence Asp Ser Gly around the reactive serine residue. It seems that there are two possible explanations for this.

Table 5. *Yields of the α, β and $\alpha\beta$ forms of Asp.SerP.Gly from di*isopropoxyphosphinyl-chymotrypsin *and from α-Asp.SerP.Gly*

Band no. (Fig. 6)	Relative yield of band in hydrolysate (method *b*, 30 min.)	
	Di*iso*propoxy-[^{32}P]phosphinyl-chymotrypsin	Band 8A
5A	36	12
8A	100	100
12	38	12

One is that the sequence is essential for enzymic activity, in which case some function must be ascribed to the aspartyl and glycyl residues for the catalytic activity, presumably in activating the serine residue. Rydon (1958) has suggested a mechanism involving the aspartyl residue, though it is more generally assumed that the activity of the serine residue is due to its spatial proximity to a specific histidine residue. No function has yet been suggested for the glycyl residue. It could perhaps be steric; for instance, it might be that a larger side chain would sterically hinder the catalytic activity. It seems possible, however, that the similarities in the structures of trypsin and chymotrypsin may extend over a larger part of the molecule (see Šorm *et al.* 1957) and this could hardly be explained on the basis of the catalytic activity. An alternative explanation is in the biological origin of the three proteins and it seems likely that they are in fact 'descended' from a common protein and have developed their specific sequences by independent mutations. Thus it may be that some more primitive organism contained a single protease and that, during the evolutionary process, three enzymes with different specificities (trypsin, chymotrypsin and elastase) have developed from it. Thus one can envisage evolution of proteins as taking place within a species, as well as between species. The nature and course of these changes could probably be revealed by a detailed study of the sequence of these and related proteins. A similar situation is found with the pituitary hormones: vasopressin and oxytocin on the one hand (du Vigneaud, Lawler & Popenoe, 1953) and adrenocorticotrophic hormone and melanophore-stimulating hormone on the other (Harris & Roos, 1956). Here again proteins or peptides from the same organ which have different biological activities have similarities in amino acid sequence and this may be expected to be a general phenomenon as more proteins are studied.

SUMMARY

1. The di*iso*propoxy[^{32}P]phosphinyl derivatives of chymotrypsin, trypsin, and elastase were subjected to partial acid hydrolysis and the products investigated after ionophoretic fractionation.

2. During acid hydrolysis an interconversion of the aspartyl peptides occurs in which the normal α form is converted into the $\alpha\beta$ and β forms.

3. By comparison of the radioactive peptides in the hydrolysates of the di*iso*propoxy[^{32}P]phosphinyl derivatives of the three enzymes it is concluded that elastase, like trypsin and chymotrypsin, contains the sequence Asp.Ser.Gly around its reactive serine residue.

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REFERENCES

Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Dixon, G. H., Kauffman, D. L. & Neurath, H. (1958). *J. Amer. chem. Soc.* **80**, 1260.
du Vigneaud, V., Lawler, H. C. & Popenoe, E. A. (1953). *J. Amer. chem. Soc.* **75**, 4880.
Edman, P. (1950). *Acta chem. scand.* **4**, 277.
Flavin, M. (1954). *J. biol. Chem.* **210**, 771.
Folsch, G. & Österberg, R. (1959). *J. biol. Chem.* **234**, 2298.
Fraenkel-Conrat, H. & Harris, J. I. (1954). *J. Amer. chem. Soc.* **76**, 6058.
Gross, D. (1955). *Nature, Lond.*, **176**, 72.
Harris, J. I. & Roos, P. (1956). *Nature, Lond.*, **178**, 90.
Hartley, B. S., Naughton, M. A. & Sanger, F. (1959). *Biochim. biophys. Acta*, **34**, 243.
John, W. D. & Young, G. T. (1954). *J. chem. Soc.* p. 2870.
Kohn, J. (1957). *Clin. chim. Acta*, **2**, 297.
Michl, H. (1951). *Mh. Chem.* **82**, 489.
Naughton, M. A. & Sanger, F. (1960). *Biochem. J.* (in the Press).
Oosterbaan, R. A., Kunst, P., Van Rotterdam, J. & Cohen, J. A. (1958). *Biochim. biophys Acta*, **27**, 549.
Partridge, S. M. & Davis, H. F. (1950). *Nature, Lond.*, **165**, 62.
Rydon, H. N. (1958). *Nature, Lond.*, **182**, 928.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 353.
Schaffer, N. K., Harshman, S. & Engle, R. R. (1955). *J. biol. Chem.* **214**, 799.
Schaffer, N. K. & Lang, R. P. (1959). *Fed. Proc.* **18**, 317.
Schaffer, N. K., Simet, L., Harshman, S., Engle, R. R. & Drisko, R. W. (1957). *J. biol. Chem.* **225**, 197.
Sjöquist, J. (1957). *Ark. Kemi*, **11**, 129.
Šorm, F., Keil, B., Holeyšorský, V., Knesslová, V., Kostka, V., Mäsiar, P., Meloun, B., Mikeš, O., Tomasek, V. & Vaněček, J. (1957). *Coll. Trav. chim. Tchécosl.* **22**, 1310.
Swallow, D. L. & Abraham, E. P. (1958). *Biochem. J.* **70**, 364.
Thompson, E. O. P. (1954). *Biochim. biophys. Acta*, **15**, 440.
Turba, F. & Gundlach, G. (1955). *Biochem. Z.* **327**, 186.

Biochem. J. (1960) **77**, 164

Ion-Exchange Reactions Between Cartilage and Various Cations

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The mechanism of calcification of cartilage is considered to involve the formation of crystal nuclei upon which epitactic growth of the bone mineral takes place (Neuman & Neuman, 1958). Although the exact nature of the agent or agents responsible for the induction of the crystal nuclei is still unknown, proposals have been made about the nature of this mineralizing site. Strates, Neuman & Levinskas (1957) have shown that collagen can produce epitactic growth of hydroxyapatite crystals, and, as a consequence, it has been suggested that collagen or some molecule associated with it (i.e. mucopolysaccharide) may be responsible for crystal induction.

DiStefano, Neuman & Rouser (1953) have suggested that the mineralizing site is anionic and that a phosphorylated polysaccharide may be a factor in the formation of crystal nuclei. The presence of this compound correlated well with the ability of cartilage to calcify *in vitro*, but the implication of this compound in the calcifying mechanism is doubtful as it has been found in various non-calcifying rat tissues (Strates, 1956).

As a result of studies on the reversible inhibition of the calcification of rachitic-bone slices by cations, Sobel (1955) suggested that chondroitin sulphate is implicated as a component of the crystal-nucleation process. Subsequently, Bachra, Sobel & Stanford (1959) and Bachra & Sobel (1959) have shown that collagen reconstituted with chondroitin sulphate or other precipitating agents is capable of mineralizing *in vitro*.

Studies by Boyd & Neuman (1951) have indicated that the binding of cations by hyaline cartilage is largely dependent on the chondroitin sulphate content of the cartilage and that the process is one of ion exchange. However, they have found that such a cartilage preparation failed to induce crystal formation at physiological concentrations of calcium and phosphate ions. From this it is concluded (Neuman & Neuman, 1958) that if Sobel is correct in assigning a role to chondroitin sulphate in crystal nucleation, then the chondroitin sulphate of actively calcifying cartilage must differ either in structure or in its combination with collagen from that of the non-mineralizing hyaline cartilage.

In further investigations of the role of chondroitin sulphate in the calcification process, Dunstone (1959) found that the capacity of cartilage for binding various cations was relatively constant for Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} ions, but higher capacities were observed for Be^{2+} and Cu^{2+} ions. The greater binding was attributed to the binding of multinuclear ions of these two elements. It was also thought that the greater binding of these two ions might be related to their powerful inhibition of calcification of rachitic-bone sections *in vitro*.

In order to study the mechanism of this inhibition of the calcification process by cations, the binding of these cationic inhibitors by cartilage has been investigated further. A study of the factors which influence the reactions between cartilage, saturated with these cations, and aqueous solutions of other cations has been made. The relative affinities of the cations for the cartilage have been measured and recorded in the form of exchange constants calculated from a Rothmund & Kornfeld (1918) equation.

Equations of this type have proved quite satisfactory in providing a quantitative measure of the relative affinities of ions for ion-exchange materials (Djurfeldt & Samuelson, 1957). Equation (1) is a modified version of the original Rothmund & Kornfeld equation for an exchange between ions of equal valency.

$$\frac{X_A}{X_B} = K\left(\frac{[A]}{[B]}\right)^p, \qquad (1)$$

where X_A and X_B are the equivalent fractions of the ions A and B in the exchanger, [A] and [B] are their concentrations in the solution, and K and p are empirical parameters.

For an exchange between an exchanger, saturated with a univalent ion, and a bivalent ion from a solution, the equation is

$$\frac{X_N}{X_M^2} = K\left(\frac{[N]}{[M]^2}\right)^p, \qquad (2)$$

where X_N and X_M are respectively the equivalent fractions of the bi- and uni-valent ions in the exchanger and [N] and [M] are their concentrations in the solution.

In the presentation of results equivalent fractions of a particular cation in the cartilage (X_c) and in the solution (X_s) are used. The equivalent fraction of a cation in the cartilage or solution phase is defined as the ratio of the number of equivalents (or m-equiv.) of that cation to the total number of

equivalents (or m-equiv.) of cations in the particular phase.

The calculation of constants from the equations (1) and (2) does not permit a direct comparison of the affinities of ions of unequal valency, although such constants do provide a good comparison between ions of the same valency. By plotting the equivalent fractions of the ions in the exchanger against their equivalent fractions in the solution, a direct comparison of the affinities of all ions, irrespective of valency, can be made (Kressman & Kitchener, 1949*a*, *b*; Bauman & Eichorn, 1947). This method has been used to compare the affinities of all ions for the cartilage.

EXPERIMENTAL

Preparation and analysis of the cation forms of cartilage. The various cation-saturated forms of the cartilage were prepared and analysed by the methods described by Dunstone (1959). All samples were analysed for both sulphate and cation.

Preparation of solutions. Cation solutions were prepared from the chlorides (A.R.) of the metals. With calcium and magnesium these chlorides were of uncertain composition and the solutions were analysed for the particular cation.

Analysis of solutions. Magnesium was determined by titration with ethylenediaminetetra-acetic acid (EDTA), Eriochrome Black T being used as indicator (Griswold & Pace, 1956). Calcium was determined by titration with EDTA, with murexide as indicator (Dunstone, 1957). Copper was determined by measuring the extinction of the copper–EDTA complex at a wavelength of 280 mμ in a Beckman model DU spectrophotometer (Dunstone, 1959).

Exchange reactions. Samples of cation-saturated cartilage (0·05–0·5 g.) were placed in glass-stoppered test tubes and measured volumes (5–100 ml.) of calcium chloride solution (6–12 m-equiv./l.) containing radioactive ^{45}Ca (40 μc/l.) were added. Shaking was carried out manually at intervals during several hours, after which time no further changes in the ion distributions occur (Dunstone, 1959). Exchange reactions involving other ions in the solution were prepared in similar fashion. No attempt has been made to control the temperature as ion-exchange reactions in general are little affected by temperature changes (Patton & Ferguson, 1937; Magistad, Fireman & Mabry, 1944; Boyd, Schubert & Adamson, 1947.)

Radiochemical analyses. After equilibrium had been reached, portions (0·2 ml.) of the supernatant solutions were transferred to aluminium planchets (25 mm. diam.), 20 drops of an asbestos suspension (Francis, Mulligan & Wormall, 1954) were added to assist spreading and the planchets were dried under an infrared lamp. The radioactivity was then measured with an end-window Geiger–Müller tube (20th century Electronics Ltd., EW3H), coupled with a standard scaling and recording unit (Philips Electrical Industries Pty. Ltd.) No corrections were necessary for self-absorption of the β-emission of ^{45}Ca, owing to the small mass on the planchets (0·4–0·7 mg.), or for the physical decay, as comparisons of initial and final concentrations were made from planchets prepared at the same time. All planchets were prepared in triplicate and provided that sufficient counts were taken (not less than 5000) the activity of the replicates did not differ by more than 2%. As a check of this method a comparison of the initial and final concentrations were made by both chemical and radiochemical methods. Identical results were obtained by both methods for the exchange between sodium-saturated cartilage and Ca^{2+} ions.

Calculations. The ratio of the initial to the final count rate, which is a measure of the ratio of the initial to the final concentration of the calcium in the solution, together with a knowledge of the mass and capacity of the cartilage, and the volume and concentration of the added solution, are sufficient data for the calculation of the concentrations of both ions in both phases. It is assumed that an equivalent exchange takes place, that no extraneous ions such as H^+ take part and that insignificant amounts of chondroitin sulphate are removed from the cartilage during equilibration.

Table 1, containing a typical set of experimental details, is included to illustrate the calculation of the results in suitable form for graphical presentation.

Exchange capacity of cartilage. The sulphate content of cartilage has been taken as a measure of the exchange capacity for the ions Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, and the complex ions of copper and beryllium (Dunstone, 1959). It is assumed that the complex ions of beryllium and copper which take part in the exchange reactions are essentially bivalent in character.

Table 1. *Calculation of experimental results*

Samples of barium-saturated cartilage (sulphate content, 0·81 m-equiv./l.) were equilibrated with 25 ml. portions of $^{45}CaCl_2$ solution (11·72 m-equiv. of Ca^{2+} ion/l., 40 μc of $^{45}Ca^{2+}$ ion/l.). After 16 hr., 0·2 ml. portions were taken for radiochemical analysis. All results are expressed as mean values of triplicate experiments. Graphical presentation of results is made by plotting the equivalent fraction of the Ca^{2+} ion in the cartilage (X_c) against its equivalent fraction in solution (X_s). The parameters, K and p, have been calculated from equation (1).

Mass of cartilage (g.)	^{45}Ca activity (counts/min./ml.)	Concn. of Ca^{2+} in soln. (m-equiv./l.)	Equivalent fraction Ca^{2+} in soln. (X_s)	Ca^{2+} bound to cartilage (m-equiv.)	Total exchange capacity of cartilage (m-equiv.)	Equivalent fraction of Ca^{2+} in cartilage (X_c)
—	4835	11·72	1·000	—	—	—
0·1	3995	9·68	0·826	0·051	0·081	0·630
0·2	3560	8·63	0·736	0·077	0·162	0·475
0·3	3190	7·74	0·660	0·100	0·243	0·412
0·4	2970	7·20	0·614	0·113	0·324	0·349
0·5	2655	6·43	0·549	0·132	0·405	0·326

RESULTS

Reversibility of the exchange reactions. The reversibility of the exchange reactions was investigated by an indirect method. $^{45}CaCl_2$ solutions (12 m-equiv./l.) were allowed to equilibrate with known masses of cation-saturated cartilage. The distribution of the ions between the phases was determined and the equivalent fraction of the Ca^{2+} ion in the cartilage phase were plotted against its equivalent

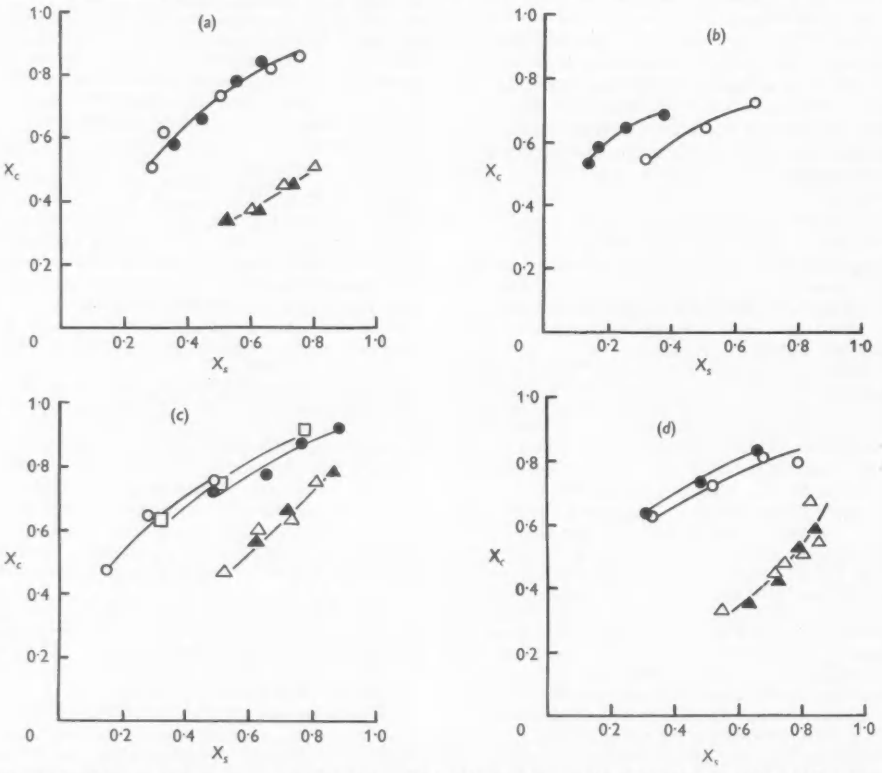

Fig. 1. Exchange reactions were carried out as described in the Experimental section. In each exchange reaction the distribution of the ions between the phases was determined (see Experimental section) and the equivalent fraction (X_c) of the Ca^{2+} ion in the cartilage phase was plotted against its equivalent fraction (X_s) in the solution phase. (*a*) Reversibility of exchange reactions. Samples (0·1–0·5 g.) of cation-saturated cartilage were equilibrated with 25 ml. portions of $^{45}CaCl_2$ soln. (12 m-equiv./l.). △, Barium-saturated cartilage; ▲, barium-saturated cartilage, 5 ml. of $BaCl_2$ soln. (12 m-equiv./l.) added after initial equilibration; ○, sodium-saturated cartilage; ●, sodium-saturated cartilage, 5 ml. of NaCl soln. (12 m-equiv./l.) added after initial equilibration. (*b*) Hysteresis effects in exchange reactions. Samples (0·1–0·5 g.) of cation-saturated cartilage were equilibrated with 25 ml. portions of cation solution (12 m-equiv./l.). ○, Sodium-saturated cartilage and $^{45}CaCl_2$ soln.; ●, calcium-saturated cartilage and NaCl soln. (*c*) Effect of solution concentration on the exchange reactions between cations and cartilage. Samples (0·1–0·5 g.) of cation-saturated cartilage were equilibrated with 25 ml. portions of $^{45}CaCl_2$ solutions of varying concentrations. ○, Sodium-saturated cartilage and $^{45}CaCl_2$ solution (7·5 m-equiv./l.); □, sodium-saturated cartilage and $^{45}CaCl_2$ solution (12·7 m-equiv./l.); ●, sodium-saturated cartilage and $^{45}CaCl_2$ solution (18·7 m-equiv./l.); △, magnesium-saturated cartilage and $^{45}CaCl_2$ solution (12·7 m-equiv./l.); ▲, magnesium-saturated cartilage and $^{45}CaCl_2$ solution (18·7 m-equiv./l.). (*d*) Effect of dilution on the exchange reactions between cations and cartilage. Samples (0·1–0·5 g.) of cation-saturated cartilage were equilibrated with 25 ml. portions of $^{45}CaCl_2$ solutions (12·7 m-equiv./l.). ○, Sodium-saturated cartilage; ●, sodium-saturated cartilage, 15 ml. of water added after initial equilibration; △, barium-saturated cartilage, 5 ml. of water added after initial equilibrium had been attained; ▲, barium-saturated cartilage, 25 ml. of water added after initial equilibration.

fraction in the aqueous phase. A second series of identical experiments were set up, and, after equilibration, portions of solutions of the cation originally present in the cartilage (12 m-equiv./l.) were added, and the system was again allowed to equilibrate. The distribution of ions between the phases was determined and the equivalent fractions were plotted on the same axes as those used for the experiments without the cation additions. Typical results are shown in Fig. 1 (*a*), which indicates that, under these experimental conditions, the exchange reactions are reversible. However, there does appear to be some slight deviation in the exchange between Ca^{2+} ions and sodium-saturated cartilage.

A second method was used to investigate further the exchanges between uni- and bi-valent ions. A solution of a bivalent ion (12 m-equiv./l.) was equilibrated with cartilage saturated with the univalent ion, and a solution of the univalent ion (12 m-equiv./l.) was equilibrated with cartilage saturated with the bivalent ion. The distributions of the ions between the phases were determined and the equivalent fractions plotted in the manner previously described. Fig. 1 (*b*) shows the results for such experiments with sodium as the univalent ion and calcium as the bivalent ion. It is obvious that different ion distributions are obtained in each case. Similar results have been found for exchanges between magnesium and sodium but with slightly greater differences between the curves.

Effect of the concentration of the solution. This effect was investigated by equilibrating $^{45}CaCl_2$ solutions of different concentrations with known masses (0·1–0·5 g.) of cation-saturated cartilage. The results of exchange reactions with sodium and magnesium as the saturating ions are shown in Fig. 1 (*c*). With decreases in solution concentration, no changes in the distribution curves were observed for the exchange between ions of equal valency, but there was an increased uptake of the bivalent ion by the cartilage for the exchanges between ions of unequal valency.

A second method of investigating this effect was also employed. Solutions of $^{45}CaCl_2$ were equilibrated with known amounts of cation-saturated cartilage, the distribution of the ions between the phases was determined and the respective equivalent fractions were plotted in the manner previously described. A second series of identical experiments were set up and, after equilibration, water was added to alter the solution concentrations. After further equilibration the ion distributions were determined and the equivalent fractions plotted on the same axes as those used for experiments without the water additions. The results shown in Fig. 1 (*d*) confirm the findings of the first method.

Affinity of cations for cartilage. The results of experiments carried out by equilibrating $^{45}CaCl_2$ solutions with cartilage saturated with the cations under investigation are shown in Fig. 2, in which the equivalent fractions are plotted as described above.

Fig. 2 clearly indicates that the order of increasing affinity for cartilage is $K^+ \doteqdot Na^+$, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Be^{2+} (probably a complex ion) and Cu^{2+} (probably a complex ion). In most experiments the mass of the cartilage was varied in preference to varying the volume of solution because a wider range of equivalent fractions in both phases could be obtained. However, identical curves were obtained when the volume of the solution was varied.

Equations (1) and (2) have been applied to the experimental data and the values for the parameters K and p are tabulated in Table 2.

The values show that the parameter p is approximately unity, that the affinity series for the bivalent ions is essentially the same as that deduced from Fig. 2 but that sodium has a slightly greater affinity for the cartilage than has potassium.

Exchange reactions were carried out in unbuffered solutions, the pH being measured at the beginning

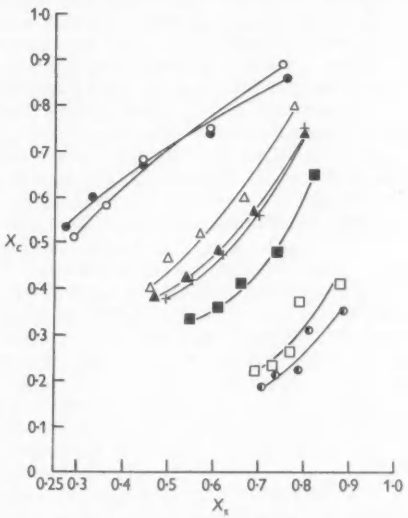

Fig. 2. Comparison of affinities of cations for cartilage. Exchange reactions were carried out and the distribution curves drawn as described in Fig. 1. Samples (0·1–0·5 g.) of cation-saturated cartilage were equilibrated with $^{45}CaCl_2$ soln. (11·7 m-equiv./l.). ○, Sodium-saturated cartilage; ●, potassium-saturated cartilage; △, magnesium-saturated cartilage; ▲, calcium-saturated cartilage; +, strontium-saturated cartilage; ■, barium-saturated cartilage; □, beryllium-saturated cartilage; ◐, copper-saturated cartilage.

and end of each experiment. The initial pH was 7·0 in all experiments. In exchange reactions involving cartilage saturated with Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} ions, the final pH varied between 6·8 and 7·2. In exchanges involving copper-saturated and beryllium-saturated cartilage, the final pH values were respectively 5·7 and 4·8.

DISCUSSION

The curves in Figs. 1–2, which have been used to represent the experimental results, may be either convex or concave. Those curves which are convex (reaction: $Ca^{2+} + Na^+$–cartilage, Fig. 2) indicate that the binding of the cation for which the equivalent fraction has been calculated (in this case Ca^{2+} ion) is stronger than that of the other reacting cation. Conversely, a concave curve (reaction: $Ca^{2+} + Ba^{2+}$–cartilage, Fig. 2) indicates that the binding of the cation for which the equivalent fraction has been calculated (in this case Ca^{2+} ion) is weaker than that of the other reacting cation. For two reacting cations that have equal strengths of binding the curve becomes a straight line joining the points (0, 0) and (1·0, 1·0). Thus if a common cation is caused to react with cartilage saturated with various cations, a series of cation-binding affinities can be determined by a study of the relative positions of the ion-distribution curves.

The curves illustrated in Figs. 1 and 2 are similar to those derived theoretically by Bauman & Eichorn (1947) and those obtained practically by Kressman & Kitchener (1949*a*, *b*) for other exchange materials. However, the curves obtained with cartilage differ from those obtained by the above-mentioned workers in two respects. First, for the exchange between a bivalent ion in solution and a cartilage-bound univalent ion the curve appears to approach a maximum value of 0·9 and not 1·0 for the equivalent fraction in the cartilage. Secondly, the curve for the reaction between Ca^{2+} ions and calcium-saturated cartilage does not follow the expected curve for an exchange involving cations of equal cation-binding affinity. These differences indicate that a fraction of the cations initially bound to the cartilage is not capable of exchanging under these experimental conditions.

It is evident from Fig. 1 (*a*) that the exchange reactions are reversible with only slight deviations in the exchange between Ca^{2+} ions and sodium-saturated cartilage.

This finding appears to be contradicted by two facts, namely: (i) the ion distribution obtained when a cartilage-bound univalent ion is exchanged for a bivalent ion is different from the ion distribution obtained when a cartilage-bound bivalent ion is exchanged for a univalent ion (Fig. 1*b*); (ii) the parameter, K, for the exchange between calcium-saturated cartilage and Ca^{2+} ions, is 0·62 (where $p \doteqdot 1$), and not unity, as would be expected from equation (1).

These results also suggest that some of the ions initially bound to cartilage are bound in such a way as to prevent their ready exchange and that those ions which exchange readily do so reversibly. Similar hysteresis effects have been observed for other naturally occurring exchange materials by Vanselow (1932).

The experimental data used in Fig. 2 to show the exchange between calcium-saturated cartilage and Ca^{2+} ions, coupled with the fact that the value of K for this reaction should be unity (for $p = 1$), should enable the amount of calcium bound 'irreversibly' to the cartilage, under these experimental conditions, to be determined. Such a calculation has been made and 18 % of the initially bound Ca^{2+} ions have been found to be bound 'irreversibly'.

The curves for exchanges in which univalent ions are initially bound to cartilage indicate that the fraction of 'irreversibly' bound ions lies in the range 10 (Figs. 1*a*, 1*c*, 1*d*, Fig. 2) to 20 % (Fig. 1*b*).

Fig. 3 has been obtained by recalculating the experimental data of Fig. 1 (*b*) assuming that 20 % of the cations initially bound to cartilage are bound 'irreversibly'. It can be seen that the data which previously defined two separate curves now define a single curve of the type expected for a thermodynamically reversible exchange reaction (Bauman & Eichorn, 1947; Kressman & Kitchener, 1949*a*, *b*). An identical result has been found for the sodium–magnesium exchange on cartilage. These facts further support the hypothesis that a fraction of cartilage–bound cations are bound 'irreversibly' under these experimental conditions.

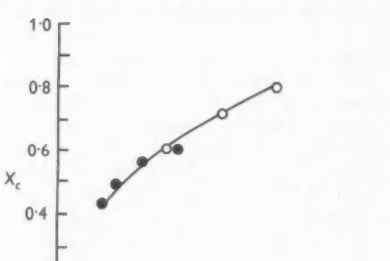

Fig. 3. Recalculation of the experimental data from Fig. 1 (*b*) on the basis that 20 % of initially bound cations are bound 'irreversibly'. ○, Sodium-saturated cartilage and $^{45}CaCl_2$ soln.; ●, calcium-saturated cartilage and NaCl soln.

Table 2. *Exchange parameters for reactions between cartilage and cations*

The parameters were calculated by applying equations (1) and (2) to the experimental data.

Reaction	Parameters calculated on the assumption that all initially bound ions are exchangeable		Parameters calculated on the assumption that 82% of the cations initially bound are exchangeable	
	K	p	K	p
$Ca^{2+} + K^{+}$–cartilage	0·09	0·86	0·10*	0·98*
$Ca^{2+} + Na^{+}$–cartilage	0·05	0·98	0·05*	1·05*
$Ca^{2+} + Mg^{2+}$–cartilage	0·83	1·03	1·35	1·06
$Ca^{2+} + Ca^{2+}$–cartilage	0·62	0·97	1·00	1·06
$Ca^{2+} + Sr^{2+}$–cartilage	0·60	0·96	0·82	1·03
$Ca^{2+} + Ba^{2+}$–cartilage	0·41	0·80	0·50	1·03
$Ca^{2+} + Be^{2+}$–cartilage	0·12	0·91	0·18	0·94
$Ca^{2+} + Cu^{2+}$–cartilage	0·09	0·95	0·11	1·03

* From Fig. 2, the exchangeable fraction in reactions involving Na^{+} and K^{+} ions approximates to 90%, and this fraction has been used in the recalculation of the results for these reactions.

By the use of this hypothesis and the experimental data of Fig. 2, the exchange parameters have been recalculated and are included in Table 2.

The estimation of the fraction of the cations bound 'irreversibly' can be regarded only as approximate, hence the recalculated parameters must also be regarded as approximate. However, these recalculated parameters may be considered as representing thermodynamically reversible reactions. The recalculation has conferred more uniformity on the parameter p, which can be considered as unity. Thus equations (1) and (2) become equivalent to mass-action equations, the parameter K representing the equilibrium constant.

Evidence that equations such as (1) and (2) can be used to describe these exchange reactions is found in Figs. 1 (*c*) and 1 (*d*), where the equation predicts and the experiment confirms that a decrease in solution concentration favours an increase in the selectivity of the cartilage for bivalent ions.

The cation-binding function of cartilage probably depends on the carboxyl and sulphate groups in the chondroitin sulphate (Dunstone, 1959). Changes of pH might be expected to modify the behaviour of such groups towards cations, but in order to avoid the introduction of competing ions the reaction mixtures were not buffered. However, the pH remained relatively constant for all exchanges except those involving beryllium and copper, where the decrease in pH was probably due to the hydrolysis of the displaced cation. In these two cases the H^{+} ion liberated in the hydrolysis may constitute a third competitive ion in the exchange reaction. No estimate of the possible contribution made by the H^{+} ion could be made, but it would appear from Fig. 2 that the contribution would have to be considerable to alter the positions of beryllium and copper relative to those of the other ions in the affinity series.

The series of elimination concentrations for cations reported by Simkiss & Tyler (1958) for cation-binding by egg-shell sulphated mucopolysaccharide is similar to the affinity series found for cartilage, but the positions of strontium and calcium, copper and beryllium are here reversed. These authors claim correlation between their elimination concentration series and the order of stability constants of the metal-chelate compounds. It is doubtful if such a correlation exists with cartilage as the order of affinity found for the alkaline-earth cations, except beryllium, is directly opposite to the order of stability of their chelate compounds (Martell & Calvin, 1952). However, the ions most strongly bound to cartilage, namely copper and beryllium, do form more stable chelates than those ions which are less strongly bound to the cartilage.

The cation-affinity series for cartilage bears some resemblance to the order of calcification-inactivating power of cations reported by Sobel (1955). The cations which inhibit the calcification process to the greatest extent, namely beryllium and copper, are among the ions most strongly bound to cartilage, whereas those which least inhibit calcification, namely sodium and potassium, show a low affinity for cartilage. With the affinity series as a basis, strontium would be expected to inhibit calcification to a greater extent than does magnesium, but Sobel (1955) has shown that its inactivating power lies somewhere between the powers of sodium and potassium. The differences observed between these two series may be due to the chondroitin sulphate of this hyaline-cartilage preparation differing structurally, or in its combination with protein, from that in the calcifying tissue used by Sobel (1955) or it may be that the process of calcification-inhibition is not one of simple ion exchange involving the 'reversible' binding sites of cartilage. The cartilage

preparation used represents a special case and samples taken from other sources and subjected to different chemical treatments might yield a different affinity series. Consequently, strict comparisons of results with those obtained by other workers should not be made.

Up to this point only the possible significance of the affinities for cartilage of 'reversibly bound' cations has been discussed. It is possible that the relative affinities of the 'irreversibly bound' cations might be of importance in the study of the mechanism of calcification, but the experimental data at present available do not permit discussion of this possibility.

SUMMARY

1. Some of the factors which influence exchange reactions between cations and cartilage have been investigated.

2. It has been found that a fraction of the cations initially bound to cartilage does not exchange readily with other cations. Those cations which do exchange readily do so reversibly.

3. The affinities of some cations for cartilage have been measured and recorded in the form of exchange constants, calculated from a Rothmund & Kornfeld (1918) equation. The order of increasing affinity for cartilage is K^+, Na^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Be^{2+} (probably a complex ion) and Cu^{2+} (probably a complex ion).

4. A comparison of this affinity series with those obtained in studies on sulphated mucopolysaccharides and on the mechanism of calcification has been made.

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REFERENCES

Bachra, B. N. & Sobel, A. E. (1959). *Arch. Biochem. Biophys.* **85**, 9.
Bachra, B. N., Sobel, A. E. & Stanford, J. W. (1959). *Arch. Biochem. Biophys.* **84**, 79.
Bauman, W. C. & Eichorn, J. (1947). *J. Amer. chem. Soc.* **69**, 2830.
Boyd, E. S. & Neuman, W. F. (1951). *J. biol. Chem.* **193**, 243.
Boyd, G. E., Schubert, J. & Adamson, A. W. (1947). *J. Amer. chem. Soc.* **69**, 2818.
DiStefano, V., Neuman, W. F. & Rouser, G. (1953). *Arch. Biochem. Biophys.* **47**, 218.
Djurfeldt, R. & Samuelson, O. (1957). *Acta chem. scand.* **11**, 1209.
Dunstone, J. R. (1957). *Med. J. Aust.* **2**, no. 16, 571.
Dunstone, J. R. (1959). *Biochem. J.* **72**, 465.
Francis, G. E., Mulligan, W. & Wormall, A. (1954). *Isotopic Tracers*, p. 137. London: The Athlone Press.
Griswold, R. L. & Pace, N. (1956). *Analyt. Chem.* **28**, 1035.
Kressman, T. R. E. & Kitchener, J. A. (1949*a*). *J. chem. Soc.*, p. 1190.
Kressman, T. R. E. & Kitchener, J. A. (1949*b*). *J. chem. Soc.*, p. 1201.
Magistad, O. C., Fireman, M. & Mabry, B. (1944). *Soil Sci.* **57**, 371.
Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*, p. 193. New York: Prentice-Hall Inc.
Neuman, W. F. & Neuman, M. W. (1958). *The Chemical Dynamics of Bone Mineral*, chap. 7. Chicago: University of Chicago Press.
Patton, J. R. & Ferguson, J. B. (1937). *Canad. J. Res.* **15**, Series B, 103.
Rothmund, V. & Kornfeld, G. (1918). *Z. anorg. Chem.* **103**, 129.
Simkiss, K. & Tyler, C. (1958). *Quart. J. micr. Sci.* **99**, 5.
Sobel, A. E. (1955). *Ann. N.Y. Acad. Sci.* **60**, 713.
Strates, B. S. (1956). M.Sc. Thesis: University of Rochester. Cited by Neuman, W. F. & Neuman, M. W. (1958). *The Chemical Dynamics of Bone Mineral*, p. 183. Chicago: University of Chicago Press.
Strates, B. S., Neuman, W. F. & Levinskas, G. J. (1957). *J. phys. Chem.* **61**, 279.
Vanselow, A. P. (1932). *Soil Sci.* **33**, 95.

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Purification of β-*N*-Acetylglucosaminidase from the Pig Epididymis

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In previous papers it has been shown that the richest source of β-*N*-acetylglucosaminidase in different mammalian species is the epididymis, and that the activity in this organ is exceptionally high in the pig (Conchie, Findlay & Levvy, 1959*a*, *b*). It is probable that this enzyme, like β-glucuronidase, is concerned in the ultimate degradation of hyaluronic acid and chondroitin (Linker, Meyer & Weissmann, 1955). Pig epididymis, like other pig tissues, displays a remarkably low β-glucuronidase

activity in comparison with the tissues of other mammalian species (Levvy & Marsh, 1959). Since pig epididymis can be obtained in quantity, it seemed a good starting material for the purification of β-*N*-acetylglucosaminidase.

EXPERIMENTAL AND RESULTS

Enzyme assay. The liberation of *p*-nitrophenol and phenol from their *N*-acetyl-β-glucosaminides was followed at 37° by the procedures described by Findlay, Levvy & Marsh (1958). With both substrates, the pH of the incubation mixture was made 4·25, to correspond to the optimum observed in the present work. As before, 0·1 M-NaCl was present in the incubation mixture and exerted the usual 5–10 % activation observed with this enzyme.

Epididymal preparations at all stages of purification showed a progressive fall in net activity with dilution, that was unaffected by NaCl. To overcome this, it was necessary to have albumin, as well as NaCl, present in the incubation mixture. Crystalline bovine-plasma albumin (L. Light and Co. Ltd.) was used, in a final concentration of 0·01 %. At the usual level of enzyme activity for assay, activation by albumin rose progressively from 10 to 100 % or more as the specific activity of the preparation increased. This effect was additive to that of NaCl. The purified enzyme at high dilution was unstable in the absence of albumin, leading to variable activation figures for the latter.

At the final stage of purification 0·02–0·05 μg. of enzyme protein was sufficient for assay with either substrate in the presence of albumin.

Protein determination. Protein was determined at all stages by the method of Lowry, Rosebrough, Farr & Randall (1951), as adapted for the Spekker photoelectric absorptiometer by Levvy, McAllan & Marsh (1958). The colour reaction was done throughout at 25°. Albumin was selected as standard. This choice was confirmed by micro-Kjeldahl determinations of N, which gave figures ranging from 15 to 17 % of the protein in stages 3 and 7*b* of the purification (Table 3). However, the whole tissue and a trichloroacetic acid precipitate from it had an N content of 21 % of the protein, estimated as albumin. It would appear therefore that the epididymis was rich in less chromogenic protein such as protamine (Levvy *et al.* 1958), and that this was removed at stage 3.

β-N-*Acetylglucosaminidase activity of different portions of the pig epididymis*

The epididymides were dissected from the testes immediately after the animal was killed and were stored at −20° until required. There was no loss in enzyme activity during storage. The organ was cut into portions before thawing, as shown in Fig. 1. After thawing at room temperature the tissue was suspended in ice-cold water by treatment in a Waring Blendor for 90 sec. (There was no apparent inactivation of the enzyme during this treatment, as compared with controls suspended with a glass homogenizer.) Table 1 shows the activity of the different portions of the epididymis. One portion of the caput epididymis had a much higher enzyme activity than had the rest of the organ, and this was a perfectly consistent phenomenon. This most active section of the caput was frequently taken for purification of the enzyme, since a high specific activity could be reached at a much earlier stage of purification (Table 3). (Rat epididymis also showed a higher β-*N*-acetylglucosaminidase activity in the caput than in the cauda.)

Table 2 records figures for a whole epididymis compared with values for other pig tissues. Apart from those for the

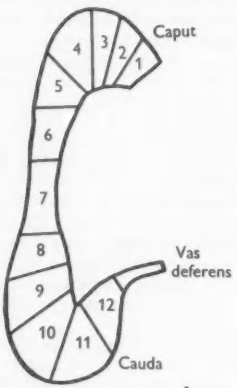

Fig. 1. Division of epididymis for assays (portion numbers shown in Table 1).

Table 1. β-N-*Acetylglucosaminidase activity of pig epididymis divided into portions as shown in Fig.* 1

Results are expressed as μg. of *p*-nitrophenol liberated from its *N*-acetyl-β-glucosaminide in 1 hr. by 1 mg. of moist tissue. Assays were in the presence of albumin.

Portion no.	Moist wt. (g.)	*p*-Nitrophenol (μg./mg. of tissue)
1	5·4	180
2	5·8	768
3	4·7	1980
4	6·1	6680
5	6·3	2620
6	5·5	712
7	4·4	540
8	4·9	448
9	8·4	550
10	18·7	1000
11	13·9	728
12	18·7	224

Table 2. β-N-*Acetylglucosaminidase activity of different pig tissues*

Results are expressed as in Table 1.

	p-Nitrophenol (μg./mg. of tissue)
Liver	44
Spleen	92
Kidney	133
Testis (adult)	48
Testis (infant)	103
Epididymis (adult)	1000–2000
Epididymis (infant)	29

adult epididymis, the figures are not strikingly different from those reported for mouse and rat organs (Conchie *et al.* 1959*a*).

Purification procedure

General comments. It was borne in mind that the procedure should be equally applicable to the whole epididymis or to selected portions. But for the variable composition of the tissue, the last two stages could have been replaced by a single fractionation. The final procedure is essentially an alternate fractionation with $(NH_4)_2SO_4$ and acetone, and Table 3 shows this applied to the whole organ and to the most active section of the caput. Ethanol was employed in early experiments but proved less reliable than acetone. The specific activity of the starting material was enhanced by its low protein content (determined as albumin), which averaged 4·5% throughout the tissue.

Precipitates were allowed to settle at 0° for 30 min. before centrifuging, which was done at 0°. The centrifuging of $(NH_4)_2SO_4$ precipitates was done at 10 000 *g* for 10 min.; acetone precipitates were centrifuged for 10 min. at 25 000 *g*. Volume changes were corrected for by adding water after mixing the reagents. Analytical-grade $(NH_4)_2SO_4$ was employed.

General manipulation of the preparation was done at room temperature. At stage 2 the homogenate was centrifuged at room temperature for 15 min. at 1500 *g*. Solutions were dialysed at 0° in Visking sausage casing for 5 days against several changes of 0·05 M-citric acid–NaOH buffer, the pH of which was altered at stage 5. At stage 3 NaCl became unnecessary for complete solution of the enzyme (cf. Pugh, Leaback & Walker, 1957*a*). There was usually a recovery at stage 7*b* of 20% of the original activity, and the purification ranged from 20-fold for the most active section of the caput to 70-fold for the whole epididymis. The specific activities attained were variable at stage 6 but tended towards the same value at stage 7. Generally the final buffered solution was clear and colourless and the concentrated enzyme was stable for several weeks at 0°.

Stage 1 (*homogenate*). The tissue was suspended in about 3 parts of ice-cold water with the Waring Blendor, and the homogenate was made 0·05 M with respect to citric acid–NaOH buffer, pH 4·3, and 0·1 M with respect to NaCl. It was incubated for 4 hr. at 37° and stored overnight at −20°.

Stage 2 (*supernatant*). After the homogenate had been thawed at 37° the insoluble material was centrifuged off and washed on the centrifuge with an equal volume of 0·05 M-buffer, pH 4·3, containing 0·1 M-NaCl. The washing fluid was added to the supernatant, which was cooled to 0°.

Stage 3 (*first ammonium sulphate fractionation*). Cold, saturated $(NH_4)_2SO_4$ solution, pH 4·3, was added to the supernatant to give a final saturation of 20% and the precipitate was discarded. The preparation was brought to 70% saturation with $(NH_4)_2SO_4$ and this precipitate was retained. It was dissolved in the minimal volume of 0·05 M-buffer, pH 4·3, and dialysed against the same buffer. Material that separated from solution was discarded.

Stage 4 (*first acetone fractionation*). Cold acetone was added stepwise to give concentrations of 20, 40 and 60% (v/v). All three precipitates were dissolved in minimal volumes of 0·05 M-buffer, pH 4·3. Any insoluble material was discarded.

Stage 5 (*second ammonium sulphate fractionation*). Fraction 4*b* was made 40, 65 and 75% saturated with $(NH_4)_2SO_4$. All three precipitates were dissolved in 0·05 M-citric acid–NaOH buffer, pH 5·5, and dialysed against the same buffer. Insoluble material was discarded.

Stage 6 (*second acetone fractionation*). Fraction 5*b* was treated with cold acetone in final concentrations of 20, 35

Table 3. *Purification of β-N-acetylglucosaminidase from pig epididymis*

Specific activities are expressed as μg. of *p*-nitrophenol liberated from its *N*-acetyl-β-glucosaminide in 1 hr. by 1 mg. of protein, assayed in the presence of albumin. Percentages of saturated ammonium sulphate solution (SAS) and acetone are by volume.

			Whole epididymis		Most active section of epididymis	
Stage	Description	pH	Specific activity	Recovery (μg. of *p*-nitrophenol/mg. of moist tissue)	Specific activity	Recovery (μg. of *p*-nitrophenol/mg. of moist tissue)
1	Homogenate	4·3	40 800	1580	139 000	6870
2	Supernatant	4·3	61 000	1270	247 000	5630
3	20–70% SAS	4·3	151 000	1110	895 000	3670
4*a*	0–20% acetone	4·3	78 700	40	750 000	85
b	20–40% acetone	4·3	308 000	782	1 600 000	3250
c	40–60% acetone	4·3	1 350	18	3 030	2
5*a*	0–40% SAS	4·3	201 000	121	749 000	225
b	40–65% SAS	4·3	670 000	583	2 240 000	2000
c	65–75% SAS	4·3	271 000	2	2 230 000	305
6*a*	0–20% acetone	5·5	500 000	2	1 380 000	2
b	20–35% acetone	5·5	2 200 000	456	3 260 000*	1860
c	35–50% acetone	5·5	41 900	18	247 000	45
7*a*	0–20% acetone	5·5	1 810 000	3	1 790 000	9
b	20–25% acetone	5·5	2 890 000	348	3 270 000	1280
c	25–30% acetone	5·5	2 390 000	51	1 770 000	16

* Lower values were usually observed here.

and 50%. The precipitates were dissolved in 0·05 M-buffer, pH 5·5, in the usual way.

Stage 7 (third acetone fractionation). Fraction 6*b* was fractionated with 20, 25 and 30% acetone, and the precipitates were dissolved in 0·05 M-buffer, pH 5·5, in which they were completely soluble.

Properties of the enzyme

Effects of varying the enzyme concentration and the duration of hydrolysis. These experiments were done at pH 4·25 with 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide in citric acid–NaOH buffer containing NaCl. The rate of hydrolysis by the purified enzyme stayed unchanged for 3 hr. in the presence of albumin, but fell rapidly in its absence after 1 hr. Hydrolysis was proportional to the amount of enzyme added when albumin was present. Without albumin, the net activity rose as more enzyme was added to the incubation mixture (see above).

Effects of pH. Fig. 2 shows that the optima for the hydrolysis of phenyl and *p*-nitrophenyl *N*-acetyl-β-glucosaminides by the purified enzyme in the presence of NaCl and albumin are identical at about pH 4·2. At the optimum, the mean ratio of *p*-nitrophenol (μg.) to phenol (μg.) liberated per mg. of protein was 2·5. Withdrawal of NaCl merely caused a slight fall in activity without any change in the pH optimum. It was impossible to determine the pH optimum in the absence of albumin, owing to the instability of the enzyme. However, a preparation of the most active section of the caput at stage 2 (Table 3), specific activity 194 000 *p*-nitrophenol units/mg. of protein, gave a curve for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosamide identical with that shown in Fig. 2, whether albumin was present or not. NaCl was present in both cases and the activation produced by albumin was 15%.

The pH optimum for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosaminide by a rat-epididymis preparation corresponding to stage 3 in Table 3 was again the same as that shown in Fig. 2, in the presence or absence of NaCl. Albumin was unnecessary with this preparation, owing to its relatively low specific activity. The optimum for the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosaminide by a ram-testis preparation is given as pH 4·4 by Heyworth, Borooah & Leaback (1957), and, for the hydrolysis of the phenyl glycoside by a rat-kidney preparation, as pH 4·3 by Pugh, Leaback & Walker (1957*b*).

Fig. 3 suggests that the shape of the pH–activity curve at low pH is determined largely by the instability of the enzyme in this region. The concentrated preparation was diluted several hundredfold with 0·01% albumin, giving a final pH of 5–6.

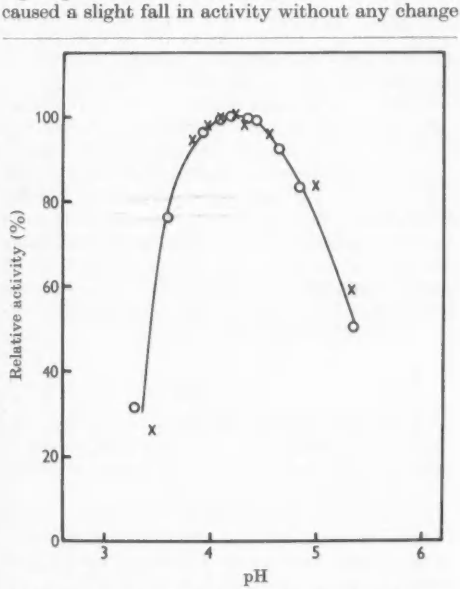

Fig. 2. Hydrolysis of 0·01 M-phenyl *N*-acetyl-β-glucosaminide (○) and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide (×) at varying pH values in 0·05 M-citric acid–NaOH buffer, in the presence of 0·1 M-NaCl and 0·01% of albumin. Specific activity of enzyme: 3 280 000 (○) and 2 740 000 (×) μg. of *p*-nitrophenol/mg. of protein respectively (cf. Table 3).

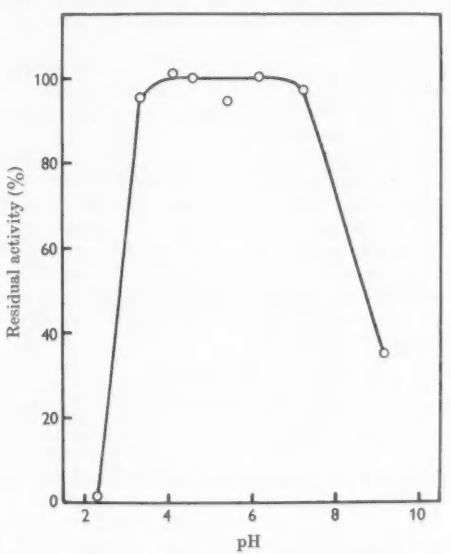

Fig. 3. Stability of the purified enzyme maintained at varying pH for 1 hr. at 37°: specific activity was 3 270 000 μg. of *p*-nitrophenol/mg. of protein (cf. Table 3).

Table 4. *Dissociation constants for the complexes formed by purified β-N-acetylglucosaminidase with substrates (K_m) and competitive inhibitors (K_i)*

Experiments were done in 0·05 M-citric acid–NaOH buffer, pH 4·25. Sodium chloride or albumin or both were added as shown. Enzyme was at stage 7*b* (Table 3), except where otherwise shown.

Substrate aglycone	0·1 M-NaCl	0·01% Albumin	K_m (mM)	K_i: *N*-Acetylglucosaminonolactone (μM)	K_i: *N*-Acetylgalactosaminonolactone (μM)
p-Nitrophenol	−	−	1·8	.	.
	+	−	2·1	.	.
	−	+	2·1	.	.
	+	+	2·1	0·46	2·0
p-Nitrophenol with enzyme at stage 3	+	+	2·0	0·51	2·1
Phenol	+	+	3·1	0·50	1·4

Specimens were adjusted to the required pH at 0° with NaOH or H_2SO_4, incubated for 1 hr. at 37°, cooled to 0° and brought back to the original pH. After further dilution, they were assayed with *p*-nitrophenyl *N*-acetyl-β-glucosaminide under optimum conditions and compared with an untreated control that had been kept at 0°. A preparation at stage 3 (Table 3) gave a similar curve, as did a rat-epididymis preparation at a corresponding stage of purification.

Dissociation constants. Table 4 gives mean values obtained with a number of different enzyme preparations for K_m for the two substrates, and for K_i *N*-acetyl-2-amino-2-deoxy-D-gluconolactone (*N*-acetylglucosaminonolactone) and *N*-acetylgalactosaminonolactone [for definition of constants, see Dixon & Webb (1958)]. Lactone solutions were prepared immediately before use. The results, calculated by the method of Lineweaver & Burk (1934), are in all respects similar to those obtained with the enzyme from rat epididymis (Findlay *et al.* 1958), except that all constants are five times as great with the pig preparations. As implied by the inhibitory action of *N*-acetylgalactosaminonolactone, our purest enzyme preparation was found to be highly active in hydrolysing *p*-nitrophenyl *N*-acetyl-β-galactosaminide in qualitative experiments.

DISCUSSION

As with purified β-glucuronidase from the female-rat preputial gland (Levvy *et al.* 1958), albumin caused considerable activation of β-*N*-acetylglucosaminidase from pig epididymis, which was not pH-dependent, and abolished the fall in net activity that accompanied dilution of the preparation. With both enzymes, K_m was the same in the presence or the absence of albumin, within experimental error. The simplest explanation of this phenomenon is an increase in the proportion of the enzyme protein in the active form (e), resulting in an increase in the rate of hydrolysis ($V = k_3 e$), without change in k_1, k_2 and k_3 in the equations:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products},$$

where E is the enzyme, S the substrate and $K_m = (k_2 + k_3)/k_1$. The same arguments, of course, apply to the small degree of activation of β-*N*-acetylglucosaminidase caused by sodium chloride.

Apart from its action on acidic mucopolysaccharides containing glucuronic acid, β-glucuronidase hydrolyses the β-glucuronides that are formed *in vivo* from alcohols, phenols and certain carboxylic acids. β-*N*-Acetylglucosaminidase, on the other hand, has no known natural substrates analogous to the β-glucuronides, but its action may be expected to extend to mucosubstances of all classes, since these always contain amino sugars, predominantly, if not entirely, *N*-acetylglucosamine and *N*-acetylgalactosamine.

SUMMARY

1. β-*N*-Acetylglucosaminidase from pig epididymis has been purified to give a final activity of about 3 million *p*-nitrophenol units/mg. of protein on assay in the presence of albumin.

2. Albumin caused extensive activation of the purified enzyme and abolished the fall in specific activity that occurred on dilution of the preparation.

The authors gratefully acknowledge the willing co-operation of Robert Lawson and Sons (Dyce) Ltd. in supplying pig epididymides, the gift of a specimen of *p*-nitrophenyl *N*-acetyl-β-galactosaminide from Dr R. Heyworth and a grant from the Medical Research Council to one of them (J. F.).

REFERENCES

Conchie, J., Findlay, J. & Levvy, G. A. (1959*a*). *Biochem. J.* **71**, 318.

Conchie, J., Findlay, J. & Levvy, G. A. (1959*b*). *Nature, Lond.*, **183**, 615.

Dixon, M. & Webb, E. C. (1958). *Enzymes*. London: Longmans Green and Co. Ltd.

Findlay, J., Levvy, G. A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 467.

Heyworth, R., Borooah, J. & Leaback, D. H. (1957). *Biochem. J.* **67**, 21P.

Levvy, G. A., McAllan, A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 22.

Levvy, G. A. & Marsh, C. A. (1959). *Advanc. Carbohyd. Chem.* **14**, 381.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Linker, A., Meyer, K. & Weissmann, B. (1955). *J. biol. Chem.* **213**, 237.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Pugh, D., Leaback, D. H. & Walker, P. G. (1957*a*). *Biochem. J.* **65**, 16P.

Pugh, D., Leaback, D. H. & Walker, P. G. (1957*b*). *Biochem. J.* **65**, 464.

Biochem. J. (1960) **77**, 175

Metabolism of Polycyclic Compounds

16. THE METABOLISM OF 1:2-DIHYDRONAPHTHALENE AND 1:2-EPOXY-1:2:3:4-TETRAHYDRONAPHTHALENE*

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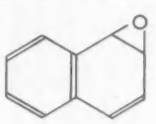
Previous work on the metabolism of naphthalene in animals has led to the suggestion that *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III) arise in the body through the same intermediate (Boyland & Sims, 1958; Sims, 1959; Booth, Boyland & Sims, 1960). The most probable structure for the intermediate would be the epoxide (I), which has not yet been prepared. The related epoxide, 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II), is available, however, and the present paper describes the metabolism of this compound and its related hydrocarbon, 1:2-dihydronaphthalene. Previous work (Pohl & Rawicz, 1919; Boyland & Solomon, 1955) indicates that 1:2-dihydronaphthalene is converted by animals into a substance which yields naphthalene on acidification, whereas the present work has shown that both 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene are con-

* Part 15: Booth, Boyland & Sims (1960).



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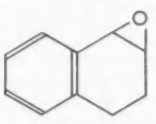

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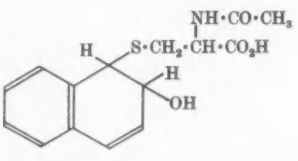
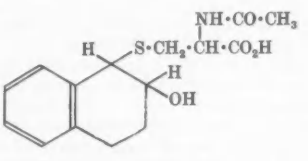


(III)



(IV)

verted into *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene and a mercapturic acid of structure (IV). Dihydroxy compounds have been isolated from the urine of animals treated with indene (Brooks & Young, 1956) and with acenaphthylene (Brooks, Hopkins & Young, 1959).

EXPERIMENTAL

Melting points are uncorrected.

Paper chromatography

Except where stated, paper chromatography was carried out by downward development for 18 hr. on Whatman no. 1 paper with the following solvents: 1, butanol saturated with aq. 2N-NH_3 soln.; 2, butanol–propan-1-ol–aq. 2N-NH_3 soln. (2:1:1, by vol.); 3, butanol–acetic acid–water (12:3:5, by vol.); 4, butanol–acetic acid–water (2:1:1, by vol.). The dried chromatograms were examined under u.v. light and then treated with one of the following reagents: freshly diazotized *p*-nitroaniline (0·02% in 0·1N-HCl) followed by aq. 10% Na_2CO_3; 0·1M-$K_2Cr_2O_7$–acetic acid (1:1) followed by 0·1M-$AgNO_3$ (Knight & Young, 1958); aq. 2% (w/v) $NaIO_4$ followed, after 30 min., by Schiff's reagent (Brooks & Young, 1956); the platinic iodide reagent of Toennies & Kolb (1951); a solution of ninhydrin in acetone (0·2%) and heating the papers to 70° for 10 min. The first three reagents were applied to the papers by spraying and the last two by dipping the papers in the reagent. The $K_2Cr_2O_7$–$AgNO_3$ reagent was better than the platinic iodide reagent in the detection of small amounts of the sulphur-containing substances, and it could be used on papers previously treated with ninhydrin. The properties of compounds related to 1:2-dihydronaphthalene are listed in Table 1.

Amino acids were detected with ninhydrin on chromatograms developed with solvents 3 and 4 described above and also by conversion into their dinitrophenyl derivatives (Sanger, 1945) followed by chromatography on Whatman no. 7 paper with the solvents described by Phillips (1958).

Materials

1:2-*Dihydronaphthalene*. This was prepared by the method of Bamberger (1895) as an oil, b.p. 31° at 0·5 mm. Hg, and was purified as described by Rowe (1921). It was shown to be free of naphthalene by infrared analysis.

2-*Bromo*-1-*hydroxy*-1:2:3:4-*tetrahydronaphthalene*. This compound was prepared by the method of Straus & Rohrbacher (1921) and formed needles from ethanol, m.p. 112°.

1:2-*Epoxy*-1:2:3:4-*tetrahydronaphthalene* (II). The above-mentioned bromhydrin was converted into the epoxide (II) as described by Straus & Rohrbacher (1921), b.p. 85° at 1 mm. Hg, m.p. 21–22°.

(±)-trans-1:2-*Dihydroxy*-1:2:3:4-*tetrahydronaphthalene*. (±)-*trans*-1:2-Dihydro-1:2-dihydroxynaphthalene was hydrogenated in methanol at room temperature and atmospheric pressure in the presence of Adams catalyst. The product separated from benzene in flat needles, m.p. 111°. The diol (100 mg.) was heated under reflux with 2N-HCl (10 ml.) for 15 min. and the cooled solution extracted with ether. Removal of the ether afforded an oil, which on warming with semicarbazide hydrochloride and sodium

Table 1. *Paper chromatography of the metabolites of 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene*

The solvents and the reagents are described in the Experimental section. The isomeric forms of the products could not be separated on paper chromatograms.

Metabolite	R_F				Colour with ninhydrin	Reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent	Colour with the $NaIO_4$–Schiff's reagent
	Solvent 1	Solvent 2	Solvent 3	Solvent 4			
S-(2-Hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine	0·23	0·57	0·53	0·72	Purple	+	Blue*
N-Acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine	0·25	0·56	0·81	0·88	.	+	Blue*
Methyl ester of *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine	0·85	0·91	0·88	0·91	.	+	Blue*
trans-1:2-Dihydroxy-1:2:3:4-tetrahydronaphthalene	0·79	0·87	0·85	0·86	.	−	Purple, turning blue
Glucosiduronate of *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene	0·04	0·31	0·44	0·59	.	−	Pink†
Metabolite, possibly a cysteinylglycine derivative	0·15	0·39	0·70	0·79	.	+	Blue*

* After 4 hr. The reaction was not sensitive to small amounts of material.

† After 15 min.

acetate in water yielded the semicarbazone of 2-tetralone in prisms (from ethanol), m.p. 193° (Found: C, 64·8; H, 6·6. Calc. for $C_{11}H_{13}ON_3$: C, 65·0; H, 6·45%).

Reaction of 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene with cysteine.* L-Cysteine hydrochloride (6 g.) and $NaHCO_3$ (3·6 g.), in water (25 ml.), were heated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) (5·8 g.) to 60° for 15 min. with stirring. The mixture was kept at 0° for some hours and the solid, which had begun to separate during the reaction, was collected (9·8 g.). It was dissolved in a minimum volume of boiling water and the crystals which separated on cooling to room temperature were recrystallized several times from aq. ethanol to yield (−)-*S*-(2-*hydroxy*-1:2:3:4-*tetrahydro*-1-*naphthyl*)-L-*cysteine* (4·1 g.) in elongated plates, m.p. 217° (decomp.), $[\alpha]_D^{22} - 30 \pm 3°$ in 0·1 N-NaOH (*c*, 0·5) (Found: C, 58·5; H, 6·5; N, 5·1; S, 11·5. $C_{13}H_{17}O_3NS$ requires C, 58·4; H, 6·4; N, 5·2; S, 12·0%). The combined mother liquors were evaporated to 50 ml. under reduced pressure and the solid which separated was recrystallized several times from aq. ethanol to yield (+)-*S*-(2-*hydroxy*-1:2:3:4-*tetrahydro*-1-*naphthyl*)-L-*cysteine* (3·9 g.) in needles, m.p. 215° (decomp.), $[\alpha]_D^{22} + 44 \pm 3°$ in 0·1 N-NaOH (*c*, 0·5) (Found: C, 58·2; H, 6·3; N, 5·2; S, 11·7%). A mixture of the two isomers had m.p. 213° (decomp.).

The cysteine derivatives (0·5 g.) were separately heated under reflux with 5 N-HCl for 15 min. and the cooled solutions extracted with ether. The ethereal extracts were evaporated to yield oils, which in each case afforded the semicarbazone of 2-tetralone as prisms from ethanol, m.p. and mixed m.p. 191–193°. The mother liquors from the hydrolyses were shown to contain cysteine by paper chromatography.

When L-cysteine hydrochloride (2 g.) and $NaHCO_3$ (0·9 g.), in water (20 ml.) (which gave a solution of pH 4·5), were warmed with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (1·8 g.) for 30 min., an oil was formed which crystallized on cooling the mixture. The solid was recrystallized from benzene to yield (±)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene (1·65 g.) in flat needles, m.p. and mixed m.p. 111°. The mother liquors were shown by paper chromatography to contain *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine.

Reaction of 2-*bromo*-1-*hydroxy*-1:2:3:4-*tetrahydronaphthalene with cysteine.* 2-Bromo-1-hydroxy-1:2:3:4-tetrahydronaphthalene (15 g.), L-cysteine hydrochloride (10·5 g.) and NaOH (9 g.), in water (100 ml.) and acetone (150 ml.), were heated under reflux for 30 min. The solution was evaporated to 100 ml. under reduced pressure, acidified to pH 4 with conc. HCl and kept at 0° overnight. The solid was collected and separated into the two isomeric cysteine derivatives described above, the (−)-isomer (6·9 g.) forming elongated plates from aq. ethanol, m.p. 218° (decomp.), $[\alpha]_D^{22} - 30 \pm 3°$ in 0·1 N-NaOH (*c*, 0·5) and the (+)-isomer forming needles (5·4 g.) from aq. ethanol, m.p. 215° (decomp.), $[\alpha]_D^{22} + 44 \pm 3°$ in 0·1 N-NaOH (*c*, 0·5). The infrared spectra of the isomers were identical with those of the respective isomers obtained in the first preparation.

Acetylation of the isomeric cysteine derivatives. The derivatives (2 g.) (prepared by either of the above methods), each in 2 N-NaOH (10 ml.), were cooled to 0° and acetic anhydride (1 ml.) added dropwise over 15 min. with stirring. The solutions were acidified to pH 4 with 2 N-HCl and kept overnight at 0°. The (−)-isomer yielded a gum which could not be crystallized, whereas the (+)-isomer yielded a *N-acetyl* derivative which formed needles from water, m.p. 188°, $[\alpha]_D^{22} + 57°$ in 0·1 N-NaOH (*c*, 0·5) (Found: C, 57·9; H, 6·3; N, 4·5; S, 10·0. $C_{15}H_{19}O_4NS$ requires C, 58·2; H, 6·2; N, 4·5; S, 10·4%). Both isomeric *N*-acetyl derivatives were esterified with diazomethane in ether. That from the (−)-cysteine derivative yielded a gum, $[\alpha]_D^{22} + 77 \pm 5°$ in $CHCl_3$ (*c*, 0·5), and that from the (+)-derivative yielded a *methyl ester* which separated from benzene–light petroleum (b.p. 60–80°) in needles, m.p. 118°, $[\alpha]_D^{22} - 59 \pm 2°$ in $CHCl_3$ (*c*, 0·5) (Found: C, 59·4; H, 6·6; N, 4·45. $C_{16}H_{21}O_4NS$ requires C, 59·4; H, 6·5; N, 4·3%). Both esters could be eluted unchanged from an alumina column with benzene–ethanol (4:1, v/v). The *N*-acetyl derivatives, when treated with acetic anhydride in pyridine, which would acetylate the hydroxyl groups, yielded gums, both of which formed spots (at R_F 0·65 in solvent 2) on paper chromatograms sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Reaction of 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene with N-acetylcysteine.* 1:2-Epoxy-1:2:3:4-tetrahydronaphthalene (II) (0·60 g.), *N*-acetylcysteine (0·68 g.) and $NaHCO_3$ (0·4 g.) in water (10 ml.) were heated to 60° with stirring. The solution was cooled to 0° and acidified to pH 4 with 5 N-HCl. The solid which separated was recrystallized from water to yield (+)-*N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (IV) (0·58 g.) in needles, m.p. and mixed m.p. 187°, $[\alpha]_D^{22} + 55 \pm 5°$ in 0·1 N-NaOH (*c*, 0·5) (Found: N, 4·45. Calc. for $C_{15}H_{19}O_4NS$: N, 4·5%). When the mother liquors were evaporated to 1 ml. under reduced pressure, an oil separated which could not be crystallized. On paper chromatograms it was indistinguishable from the mercapturic acid (IV).

Acid-hydrolyses of the cysteine and N-acetylcysteine derivatives. About 5 mg. of each derivative was heated to 100° with 2 N-HCl (1 ml.) for 5 min. The solution was cooled and an excess of 10 N-NaOH, ethanol (0·5 ml.) and ether (0·5 ml.) were added. On shaking a blue colour developed in the ether layer. This test is specific for 2-tetralone (Straus & Rohrbacher, 1921). All the cysteine and *N*-acetylcysteine derivatives mentioned above as well as *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene gave positive reactions in this test.

The solutions obtained after heating the derivatives with conc. HCl for 4 hr. at 100° were examined for amino acids, either directly or after conversion into the 2:4-dinitrophenyl derivatives. All the cysteine and *N*-acetylcysteine derivatives described above gave rise mainly to a substance indistinguishable from cysteine, but on chromatograms treated with ninhydrin a number of small unidentified ninhydrin-positive spots were also found. All the derivatives gave rise to the same pattern of spots. If HBr (sp.gr. 1·7) was used in place of conc. HCl in the hydrolysis the numbers and sizes of these unidentified spots were decreased. Cystine was also detected as the 2:4-dinitrophenyl derivatives in these hydrolyses, but it might well be an artifact, since cysteine itself is partly converted into cystine under the conditions used.

Hydrogenolysis with Raney nickel catalyst. The *N*-acetylcysteine derivatives (IV) (50 mg.), in ethanol (10 ml.), were each heated under reflux with Raney nickel catalyst (W-2 grade) (500 mg.) for 2 hr. The Raney nickel was filtered off and the filtrates were diluted with water (50 ml.) and extracted with ether (2 × 20 ml.). The presence of a substance indistinguishable from 2-naphthol in the ether extracts was demonstrated by means of paper chromato-

graphy with the solvents and colour reactions described by Sims (1959). The aqueous phases were evaporated to dryness and the residues heated to 100° for 4 hr. with aq. HBr (sp.gr. 1·7). The solutions were evaporated to dryness and the presence of a substance indistinguishable from alanine in the residues was shown by paper chromatography, both directly and after conversion into a 2:4-dinitrophenyl derivative. No other amino acids were detected.

When *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III) [isolated from the urine of rabbits treated with naphthalene (Boyland & Sims, 1958)] was similarly treated with Raney nickel catalyst followed by extraction with ether, 2-naphthol (but no 1-naphthol) was detected in the ether extract and the residue in the aqueous layer gave rise after hydrolysis with HBr to a substance indistinguishable from alanine.

Animal experiments

A rabbit (body wt. approx. 2 kg.) was given 1:2-dihydronaphthalene (0·5 g.) in arachis oil (0·5 ml.) daily for 12 consecutive days by intraperitoneal injection. Two rabbits were each given 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) (200 mg.) in arachis oil (1 ml.) daily for 10 consecutive days. Six rats (body wt. approx. 200 g.) were each given 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) (40 mg.) in arachis oil (100 ml.) daily for 10 days. In each experiment the urines were collected separately from the faeces, pooled and stored at 0°. The pooled urines were separately filtered, acidified to pH 4 with acetic acid, and activated charcoal (100 g.) was added with stirring. The charcoal was filtered off, and washed first with water (2 l.) and then with methanol (2 l.) containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) followed by methanol–benzene (95:5, v/v) (1 l.). The methanol and methanol–benzene washings were combined and evaporated under reduced pressure. The gums thus obtained were chromatographed on cellulose-powder columns, each prepared from Whatman standard-grade cellulose powder (500 g.), with butanol–*cyclo*hexane–aq. 2N-NH_3 (9:2:1) as the developing solvent. Fractions of 250 ml. were collected, evaporated under reduced pressure and the residues (after examination on paper chromatograms) were treated as described below.

RESULTS

Examination on paper chromatograms of the urines and of the gums obtained after the treatment of the urines with charcoal showed that animals treated with either 1:2-dihydronaphthalene or with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) contained substances indistinguishable from *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (IV), *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine and a third substance which gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent. The urine from the rabbit treated with 1:2-dihydronaphthalene also contained all the naphthalene metabolites reported in previous papers (Boyland & Sims, 1958; Sims, 1959). A portion of the urine of animals injected with 1:2-dihydronaphthalene yielded small amounts of naphthalene after acidification with hydrochloric acid, but none could be detected in the acidified urines of animals treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene.

Metabolites of 1:2-*dihydronaphthalene*

Fractions 1 and 2 formed gums which contained both *trans*-1:2-dihydro-1:2-dihydroxynaphthalene (in small amount) and *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, together with a little 1- and 2-naphthol. The gums were combined and extracted three times with boiling *cyclo*hexane (25 ml.) and the combined extracts evaporated to 10 ml. volume. The crystals (25 mg.) which separated were recrystallized from benzene to yield (±)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene (11 mg.) in needles, m.p. and mixed m.p. 111°. The mother liquors yielded a small amount of material of m.p. 113–114° which did not depress the m.p. of (−)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene isolated from the urine of rabbits treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (see below). Fractions 2 and 3 contained a little 1-naphthyl sulphate together with the isomeric sulphuric esters of 1:2-dihydroxynaphthalene. Fraction 3 also contained a small amount of a substance indistinguishable from 1-naphthylmercapturic acid on paper chromatograms.

Fractions 4–7 contained the substance believed to be the mercapturic acid (IV). The fractions were combined and extracted with boiling water (3 × 10 ml.). The aqueous extracts were combined and evaporated to dryness and the residue was extracted with boiling chloroform (2 × 25 ml.). Removal of the chloroform afforded a gum (1·2 g.), which gave one spot on paper chromatograms indistinguishable from the mercapturic acid (IV). When a little of the gum was heated with 2N-HCl for 5 min. the test of Straus & Rohrbacher (1921) for 2-tetralone was positive and paper chromatography revealed the same pattern of ninhydrin-positive spots as those given by the synthetic materials. Hydrogenolysis with Raney nickel catalyst as described above yielded substances indistinguishable from 2-naphthol and alanine. When the gum was treated with acetic anhydride in pyridine, the product obtained was indistinguishable on paper chromatograms from the materials obtained by a similar treatment of the synthetic compounds. Esterification of the gum with diazomethane in ether, followed by chromatography of the product on an alumina column and the elution of the column with benzene–ethanol (4:1, v/v), yielded a gum, $[\alpha]_D^{22} + 83 \pm 5°$ in $CHCl_3$ (*c*, 0·5), which was indistinguishable on paper chromatograms from the synthetic methyl esters described above. The infrared spectrum (measured in carbon tetrachloride) of this gum was identical with those of the two synthetic methyl esters.

Fractions 8–10 formed gums which were shown by paper chromatography to contain substances indistinguishable from *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III) and *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine. Attempts to isolate the latter compound from the combined fractions were unsuccessful, but a little of the gum after heating to 100° with 2N-HCl for 5 min. gave a positive reaction in the Straus & Rohrbacher test for 2-tetralone.

Fractions 12–16 all contained a substance which gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent and negative ninhydrin reactions on paper chromatograms. The combined material from these fractions was applied along the base lines of Whatman no. 3MM chromatography paper and the chromatograms were developed for 18 hr. with solvent 3. The appropriate areas of the chromatograms were cut out and the new metabolite was eluted with hot water. Removal of the water under reduced pressure afforded a gum (80 mg.) which gave only one spot detectable with the $K_2Cr_2O_7$–$AgNO_3$ reagent. After being heated for 15 min. at 100° with 2N-HCl, the gum gave a positive test for 2-tetralone. The gum failed to react with 2:4-dinitrofluorobenzene, but after being heated with HBr (sp.gr. 1·7) at 100° for 4 hr. yielded substances on paper chromatograms indistinguishable from cysteine and glycine. When the hydrolysate was treated with 2:4-dinitrofluorobenzene, substances indistinguishable from the 2:4-dinitrophenyl derivatives of cysteine and glycine were detected on paper chromatograms. Similarly, hydrogenolysis with Raney nickel catalyst yielded a substance which did not react with 2:4-dinitrofluorobenzene, but which, after hydrolysis with HBr, yielded substances indistinguishable from alanine and glycine, and the products from the reaction of the hydrolysate with 2:4-dinitrofluorobenzene yielded compounds indistinguishable from the derivatives of these amino acids. A substance indistinguishable from 2-naphthol was also detected in the products from the hydrogenolysis. This evidence suggests that the substance is related to *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteinylglycine.

Fractions 17–26 were combined and evaporated to about 20 ml. under reduced pressure, when crystals (170 mg.) separated. These were recrystallized from aq. ethanol to yield the ammonium salt of a *glucosiduronic acid* of (+)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene in plates, m.p. 233° (decomp.), $[\alpha]_D^{22} - 74 \pm 2°$ in water (*c*, 0·5) (Found: C, 53·6; H, 6·7. $C_{16}H_{23}O_8$ requires C, 53·8; H, 6·5%). The salt gave a positive naphtharesorcinol test and a positive test for 2-tetralone after being heated with 2N-HCl. The salt was treated with β-glucuronidase at 37° in acetate buffer (pH 4·5) for 20 hr. and the reaction mixture extracted with ether to yield (+)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, separating from benzene in plates, m.p. 115°, $[\alpha]_D^{22} + 58 \pm 5°$ in ethanol (*c*, 0·5). The mother liquors, which contained small amounts of the glucosiduronic acids of *trans*-1:2-dihydro-1:2-dihydroxynaphthalene and of 1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, were evaporated to dryness, the residue was dissolved in acetate buffer (pH 4·5) and treated with β-glucuronidase as before to yield a product which appeared to be mainly (+)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene (75 mg.), separating from benzene in plates, m.p. 112–113°, $[\alpha]_D^{22} + 26 \pm 5°$ in ethanol (*c*, 0·5). The mother liquors were shown by paper chromatography to contain *trans*-1:2-dihydro-1:2-dihydroxynaphthalene.

Metabolites of 1:2-*epoxy*-1:2:3:4-*tetrahydronaphthalene*

The gums from fractions 1–3 of the urine of rabbits treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) were combined and extracted four times with boiling light petroleum (b.p. 80–100°) (20 ml.). The solid which separated from the fractions on cooling was recrystallized three times from benzene to yield (±)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene (110 mg.) in flat needles, m.p. and mixed m.p. 111°, $[\alpha]_D^{22}$ 0° in ethanol (*c*, 0·5) (Found: C, 73·2; H, 7·5. Calc. for $C_{10}H_{12}O_2$: C, 73·1; H, 7·4%). The combined light petroleum and benzene mother liquors were evaporated to 5 ml. and the solid which separated was recrystallized three times from benzene to yield (−)-*trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene (85 mg.) in plates, m.p. 115°, $[\alpha]_D^{22} - 63°$ in ethanol (*c*, 0·5) (Found: C, 73·5; H, 7·6%). Booth & Boyland (1949) report m.p. 113°, $[\alpha]_D^{22} - 62°$ in ethanol (*c*, 0·5–1) for this isomer of the diol. The infrared spectra of the two isomers, measured in carbon tetrachloride, were identical.

Fractions 6–8 were combined and evaporated and the residues extracted three times with boiling water (25 ml.). The extracts deposited, on cooling, an unidentified acid which formed pale-yellow needles (95 mg.) from water, m.p. 231°. The mother liquor, which contained a substance indistinguishable from the mercapturic acid (IV), was evaporated and the residue twice extracted with boiling $CHCl_3$ (25 ml.). Removal of the $CHCl_3$ afforded a gum (1·1 g.) which was indistinguishable in all the tests described above from the corresponding product obtained from the 1:2-dihydronaphthalene urine. The gum yielded, with diazomethane in ether, a methyl ester $[\alpha]_D^{22} + 79 \pm 5°$ in $CHCl_3$ (*c*, 0·5), giving an infrared spectrum identical with those of the synthetic products.

Fractions 9–13 were combined and evaporated to about 50 ml. and the product (175 mg.) which separated was recrystallized from water to yield an unidentified acid in prisms, m.p. 257° (decomp.). The fractions were also shown by paper chromatography to contain a substance indistinguishable from *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine.

Fractions 14–15 on evaporation yielded a gum which contained a substance giving a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent, which was indistinguishable on paper chromatograms from the unidentified metabolite of 1:2-dihydronaphthalene which appears to be related to *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteinylglycine. The substance was obtained as a clear gum (50 mg.) by paper chromatography on 3MM paper and gave tests for 2-tetralone, glycine and cysteine on acid hydrolysis, and for 2-naphthol, glycine and alanine after hydrogenolysis with Raney nickel catalyst.

Fractions 17–24 all contained a substance which was indistinguishable on paper chromatograms from the glucosiduronic acid of 1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, but the compound was not isolated.

The urine of rats treated with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene was not examined in detail, but compounds were detected in the various fractions from the cellulose column which were indistinguishable from *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, the mercapturic acid (IV) and the unidentified cysteinylglycine derivative described above. A small amount of (−)-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine, m.p. 217° (decomp.), was isolated from one of the fractions: the infrared spectrum was identical with that of the synthetic isomer.

DISCUSSION

The reaction of cysteine with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) appears to give mainly two stereoisomeric forms of a product which exist by virtue of the asymmetric carbon atom in the cysteine residue. Examination of the crude reaction mixture on paper chromatograms showed that a small amount of a ninhydrin-negative substance, which could be the product of the reaction of either the cysteine carboxyl or amino group with the epoxide, was present. Although the addition of the cysteine residue takes place in the 1-position, the possibility of some 2-addition is not ruled out since small amounts of the 2-isomer might be difficult to detect in the presence of large amounts of the 1-isomer. The structures of the isomeric forms of the cysteine derivative are confirmed by the formation of 2-tetralone and cysteine on acid hydrolysis and by the detection of 2-naphthol and alanine after the hydrogenolysis of the corresponding *N*-acetyl derivatives with Raney nickel catalyst. At pH 7 the reaction of an epoxide with a SH group proceeds more readily than reactions with carboxyl or amino groups. At pH 4·5, however, the formation of diol is the main reaction. Other epoxides, such as 1:2-epoxyindene, 1:2-epoxy*cyclo*hexane, 2:3-epoxy-1:2:3:4-tetrahydronaphthalene and epichlorhydrin all react with cysteine to give analogous cysteine derivatives (E. Boyland & P. Sims, unpublished work). The production of a 1-substituted 1:2:3:4-tetrahydronaphthalene derivative by the reaction of 2-bromo-1-hydroxy-1:2:3:4-tetrahydronaphthalene with cysteine probably proceeds through the intermediate formation of the epoxide (II). The hydroxyl group and the cysteine residue could be either *cis* or *trans* in respect to each other: since the epoxide (II) with water yields a *trans* product, the product with cysteine is probably also *trans*.

1:2-Dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene are converted in the body into both the stereoisomers of *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene. Since the glucosiduronic acid of only the (−)-isomer has been isolated or detected, whereas the free diol is found both as the (+)-form and the racemate, it is probable that the first-formed diol is in fact the racemate, which would be expected from the non-enzymic opening of the oxiran ring. There is, however, little doubt that both 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene form only one of the two possible isomeric forms of the mercapturic acid (IV) in the body. Experience with a mixture of equal amounts of both forms of the mercapturic acids has shown that that derived from (+)-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-cysteine can be separated easily from that derived from the (−)-form of the cysteine derivative. The fact that the metabolic production of the mercapturic acid derivatives leads to the formation of only one of the possible isomers indicates that the reaction in which some sulphur compound combines with the epoxide is probably enzymic.

The hydrogenolysis with Raney nickel catalyst of the mercapturic acids (III) (derived from naphthalene) and (IV) (derived from 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene) to yield in each case 2-naphthol and, after hydrolysis, alanine, provides additional confirmation of their proposed structure and demonstrates the close relationship of the two metabolites. The metabolites differ in their reaction towards mineral acid, the mercapturic acid (III) being broken down rapidly in the cold to give mainly 1-naphthylmercapturic acid (by the loss of the elements of water) (Boyland & Sims, 1958), whereas the mercapturic acid (IV) is only decomposed by hot acid

to give 2-tetralone (by the loss of the *N*-acetylcysteine residue). In these reactions the mercapturic acids resemble the corresponding dihydroxy compounds, *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, yielding mainly 1-naphthol, and *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene yielding 2-tetralone.

The presence of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine in the urine of animals treated with 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene is unexpected, since the urine of animals treated with naphthalene does not contain the related cysteine derivative, *S*-(1:2-dihydro-1:2-dihydroxy-1-naphthyl)-L-cysteine but only the mercapturic acid (III) (an acetylcysteine derivative). The third metabolite, detected in the urine of animals treated with 1:2-dihydronaphthalene, which yielded 2-tetralone, cysteine and glycine on acid hydrolysis and 2-naphthol, alanine and glycine after hydrogenolysis with Raney nickel catalyst, appears to be a derivative of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteinylglycine. *S*-Substituted cysteine and cysteinylglycine derivatives have been detected in the bile of rats dosed with either 1:2-dihydronaphthalene or naphthalene (E. Boyland & P. Sims, unpublished work) and probably arise from the breakdown of the corresponding *S*-substituted glutathione derivatives.

The unidentified acids of m.p. 231° and 257° (decomp.) isolated from the urine of rabbits dosed with 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (II) appear to be related chemically. It is not certain that they are metabolites since they were not found in the urine of rats treated with the epoxide (II) or in that of rabbits treated with 1:2-dihydronaphthalene. Their structure is now under investigation.

The naphthalene metabolites detected in the urine of animals treated with 1:2-dihydroxynaphthalene probably arise through dehydrogenation of the dihydro compound in the body. Such dehydrogenations to aromatic compounds are well known. From a comparison of the sizes of the spots produced by the various metabolites on paper chromatograms it is estimated that 10–15% of the administered dihydronaphthalene is metabolized in this way. The substance which gave naphthalene with mineral acid could not be detected among the products from the cellulose-powder column.

It will be seen therefore that there are remarkable similarities between the metabolism of 1:2-epoxy-1:2:3:4-tetrahydronaphthalene and of 1:2-dihydronaphthalene on the one hand and between 1:2-dihydronaphthalene and naphthalene on the other, and it is probable that the epoxide (II) is an intermediate in the metabolism of 1:2-dihydronaphthalene. These similarities are in agreement with the hypothesis that the as yet unknown 1:2-dihydronaphthalene-1:2-epoxide is the actual intermediate in the metabolic conversion of naphthalene either into the corresponding diol or into mercapturic acid. Further evidence that this is so is presented in the following paper.

SUMMARY

1. The action of L-cysteine on 1:2-epoxy-1:2:3:4-tetrahydronaphthalene in neutral solution or on 2-bromo-1-hydroxy-1:2:3:4-tetrahydronaphthalene in alkaline solution yielded in each case the two isomeric forms of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine. The epoxide and *N*-acetylcysteine similarly gave two isomeric forms of *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine, which were also obtained by the acetylation of the cysteine derivatives.

2. Rabbits treated with either 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene excrete both the optical isomers of *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene and one of the two isomers of *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine, together with small amounts of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine and a substance believed to be a *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-cysteinylglycine derivative. A glucosiduronic acid of *trans*-1:2-dihydro-1:2:3:4-tetrahydronaphthalene was also present.

3. Small amounts of all the known naphthalene metabolites were also present in the urine of rabbits after administration of 1:2-dihydronaphthalene.

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REFERENCES

Bamberger, S. (1895). *Liebigs Ann.* **288**, 75.
Booth, J. & Boyland, E. (1949). *Biochem. J.* **44**, 361.
Booth, J., Boyland, E. & Sims, P. (1960). *Biochem. J.* **74**, 117.
Boyland, E. & Sims, P. (1958), *Biochem. J.* **68**, 440.
Boyland, E. & Solomon, J. B. (1955). *Biochem. J.* **59**, 518.
Brooks, C. J. W., Hopkins, R. P. & Young, L. (1959). *Biochem. J.* **71**, 28P.
Brooks, C. J. W. & Young, L. (1956). *Biochem. J.* **63**, 264.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
Pohl, J. & Rawicz, M. (1919). *Hoppe-Seyl. Z.* **104**, 95.
Rowe, J. (1921). *J. Soc. chem. Ind. Lond.*, **40**, 58T.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sims, P. (1959). *Biochem. J.* **73**, 389.
Straus, F. & Rohrbacher, A. (1921). *Ber. dtsch. chem. Ges.* **54**, 40.
Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.

Metabolism of Polycyclic Compounds

17. THE REACTION OF 1:2-DIHYDRONAPHTHALENE AND 1:2-EPOXY-1:2:3:4-TETRAHYDRONAPHTHALENE WITH GLUTATHIONE CATALYSED BY TISSUE PREPARATIONS

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Naphthalene is converted into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione by rat-liver slices (Booth, Boyland & Sims, 1960). When 1:2-dihydronaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene is administered to rabbits, a mercapturic acid, *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (III), is excreted in the urine (Boyland & Sims, 1960); this product is closely related in structure to the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine found in the urine of rabbits treated with naphthalene (Boyland & Sims, 1958). The present work has shown that rat-liver slices convert 1:2-dihydronaphthalene into *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I), which is converted into *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (II) by rat-kidney homogenates and to the mercapturic acid (III) by kidney slices. The action of a rat-liver microsomal system on 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene is also described. Evidence is presented that glutathione is activated by an enzyme or enzyme system, but that cysteine and *N*-acetylcysteine are not.

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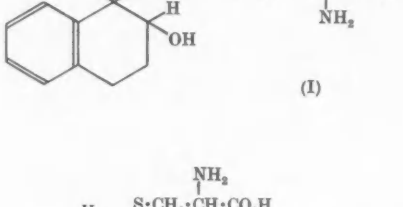
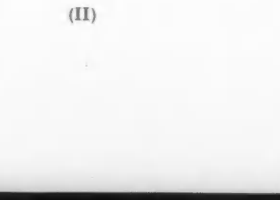
EXPERIMENTAL

Materials. 1:2-Dihydronaphthalene, 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (II) and *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (III) were prepared as described by Boyland & Sims (1960). Reduced glutathione was obtained from Schwarz Laboratories Inc.

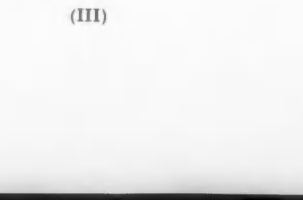
The glutathione derivative (I) was prepared as follows: 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (1 g.), glutathione (2·1 g.) and $NaHCO_3$ (0·6 g.), in water (100 ml.), were kept at 60° with stirring for 2 hr. The solution was cooled, acidified to pH 4 with acetic acid and activated charcoal (10 g.) was added. The charcoal was filtered off and washed with water (1 l.) and the adsorbed material eluted with methanol (500 ml.) containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). The methanol was removed under reduced pressure to give a clear gum which was chromatographed on a cellulose-powder column (100 g., Whatman standard grade) with butanol–*cyclo*hexane–water (18:1:3, by vol.) as developing solvent. Fractions of 200 ml. were collected,



(I)



(II)



(III)

evaporated to dryness under reduced pressure and the residue were examined for the glutathione derivative (I) on paper chromatograms. Some of the fractions containing the glutathione derivative (I) formed uncrystallizable gums, whereas others yielded colourless solids. These solids were combined, dissolved in methanol and precipitated with ether to give a product which was presumably a mixture of the isomeric forms of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I) as a colourless powder, melting over the range 130–165° (Found: N, 9·6; S, 6·9. Calc. for $C_{20}H_{27}O_7N_3S$: *N*, 9·3; S, 7·1%). A little of the derivative was heated to 100° for 5 min. with 2N-HCl (1 ml.) and the solution cooled and an excess of 10N-NaOH, ethanol (0·5 ml.) and ether (0·5 ml.) were added. On shaking, a blue colour developed in the ether layer, characteristic of 2-tetralone (Straus & Rohrbacker, 1921). A little of the derivative was heated with an excess of HBr (sp.gr. 1·7) for 4 hr. at 100°. The solution was evaporated to dryness and the residue examined on paper chromatograms for amino acids, both directly with solvent 1 described below, and, after treatment with 2:4-dinitrofluorobenzene (Sanger, 1945), with the solvents of Phillips (1958). Compounds indistinguishable from glycine, glutamic acid and cysteine (and cystine) were detected. Hydrogenolysis of the derivative (I) with Raney nickel catalyst as described by Boyland & Sims (1960) led to the detection of compounds indistinguishable from 2-naphthol, glycine, alanine and glutamic acid.

Paper chromatography. This was carried out by downward development for 18 hr. on Whatman no. 1 paper with either solvent 1, butanol–acetic acid–water (12:3:5, by vol.), or solvent 2, butanol–propan-1-ol–water (2:1:1, by vol.), as developing solvent. The properties on paper chromatograms of most of the compounds encountered in the present work have been described by Boyland & Sims (1960). *S*-(2-Hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I) had R_F 0·38 and 0·25 on paper chromatograms in solvents 1 and 2 respectively and gave a purple colour with ninhydrin and a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent of Knight & Young (1958). 2-Naphthol was detected as described by Sims (1959).

Tissue experiments. Slices of liver (approx. 2·5 g. wet wt.) from male adult rats were incubated in Krebs–Ringer phosphate (pH 7·4) (Umbreit, Burris & Stauffer, 1949) (30 ml.), containing a suspension of 1:2-dihydronaphthalene, for 2 hr. at 37° with shaking. At the end of the incubation period, the reaction mixture was decanted from the slices, which were washed once with water (15 ml.). The washings and the reaction mixture were combined and adjusted to pH 4 with acetic acid. The mixture was centrifuged for 20 min. at 600 *g*, the supernatant was treated with activated charcoal (0·5 g.) and the charcoal filtered off and washed with water. The material, obtained by the subsequent elution of the charcoal with methanol containing 0·2% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) and evaporation under reduced pressure, was examined on paper chromatograms. The kidney-slice experiments were carried out in the same way with slices (approx. 5 g. wet wt.) from the kidneys of male adult rats. Livers and kidneys from male adult rats were homogenized in 3 vol. of Krebs–Ringer phosphate (pH 7·5) with a homogenizer as described by Potter & Elvehjem (1936) with a Teflon pestle.

Rat-liver microsomes were prepared as described by (Booth & Boyland, 1958) except that the livers were homogenized in 4 vol. of 0·1M-phosphate buffer (pH 7·4), prepared from Na_2HPO_4 and KH_2PO_4. The microsomes were suspended in the original volume of iso-osmotic NaCl and reprecipitated by spinning for 30 min. at 90 000 *g* in a Spinco model L preparative ultracentrifuge with rotor no. 40. The microsomal pellets were stored at −20°. The supernatant obtained after the separation of the microsomes was adjusted to pH 5 with acetic acid, spun at 1000 *g* for 15 min. and the pH of the resulting supernatant readjusted to 7·4 by the addition, first of 5N-NaOH and finally of 0·1N-NaOH. Immediately before use a microsomal pellet was suspended in 0·1M-phosphate buffer, pH 7·4, with a Potter & Elvehjem homogenizer so that 1 ml. of suspension corresponded to 800 mg. of liver. The complete microsome system used in these experiments contained 1 ml. of microsomal suspension, 1 ml. of supernatant, 1·2 mM-nicotinamide, 0·12 mM-triphosphopyridine nucleotide (TPN), 1·2 mM-glucose 6-phosphate (G 6-P) and 0·01% of G 6-P dehydrogenase in a total volume of 10 ml. of 0·1M-phosphate buffer, pH 7·4. The solutions were saturated with 1:2-dihydroxynaphthalene or 1:2-epoxy-1:2:3:4-tetrahydronaphthalene and glutathione (0·02 mM) or cysteine HCl (0·02 mM) for 1:2-dihydronaphthalene, and glutathione (0·01 mM) or cysteine HCl (0·01 mM) for 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, added as indicated below. Other experiments were carried out in the absence of supernatant and in the absence of TPN and its reducing system. The mixtures were incubated for 15 min. at 37° with shaking, the pH of the solution was adjusted to 4·5 with acetic acid and the solutions were centrifuged at 600 *g*. The supernatants were treated with charcoal as described above, the methanolic extracts evaporated and approximately equal amounts of the residue were spotted on to paper chromatograms.

RESULTS

Preliminary experiments, in which either glutathione or cysteine in 0·1M-phosphate buffer, pH 7·4, was shaken with 1:2-dihydronaphthalene at 37° for 2 hr., showed that the glutathione derivative (I) and the cysteine derivative (II) respectively were formed in sufficient quantity to be detected on paper chromatograms. In all the tissue experiments involving 1:2-dihydronaphthalene described below, therefore, control experiments with 1:2-dihydronaphthalene and boiled tissue in 0·1M-phosphate buffer were performed at the same time and under the same conditions as in the experiments with fresh tissue. Chromatographic examination of the products of the incubation of 1:2-dihydronaphthalene with liver slices showed the presence of three metabolites which were indistinguishable from *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I), *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene and 2-naphthol, whereas the control experiments showed none of these compounds. On some occasions, *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione [previously reported as a product of the action of liver slices on naphthalene (Booth *et al.* 1960)] was

also detected. The addition of glutathione to liver slices did not result in an increased formation of the glutathione derivative (I) (presumably because the tissue had sufficient glutathione) and the addition of cysteine did not yield any of the cysteine derivative (II). When the glutathione derivative (I) was itself incubated with liver slices or with liver homogenate, neither the cysteine derivative (II) nor the *N*-acetylcysteine derivative (III) was formed.

The glutathione derivative (I) formed in the incubation of 1:2-dihydronaphthalene with liver slices was eluted from paper chromatograms with methanol. The material obtained by the evaporation of the solvent was hydrolysed by heating with conc. HCl to 100° for 4 hr. and the products of the hydrolysis were treated with 2:4-dinitrofluorobenzene (Sanger, 1945). When the derivatives were examined on paper chromatograms with the solvents of Phillips (1958), compounds indistinguishable from the 2:4-dinitrophenyl derivatives of cystine, glycine and glutamic acid were detected.

The synthetic glutathione derivative (I), on incubation with rat-kidney homogenate, was converted into a substance indistinguishable from the cysteine derivative (II), and, under the same conditions, the *N*-acetylcysteine derivative (III) was also hydrolysed, although less readily, to the same derivative (II). On the other hand, both the glutathione derivative (I) and the cysteine derivative (II) were converted by rat-kidney slices into the acetylated derivative (III), and this compound, together with *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene was also obtained when 1:2-dihydronaphthalene was incubated with kidney slices in the presence of glutathione. When boiled kidney slices were incubated with 1:2-dihydroxynaphthalene and glutathione, the dihydroxy compound, the *N*-acetyl derivative (III) and the glutathione derivative (I) were not detected.

In the experiments with rat-liver microsomes, control experiments were carried out in which 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene were incubated with either glutathione or cysteine in the presence of boiled microsomes in 0·1 M-phosphate buffer, pH 7·4, and also in 0·1 M-phosphate buffer alone. In the experiments with 1:2-dihydronaphthalene, only small amounts of the sulphur compounds and no dihydroxy compound were detected. When 1:2-dihydroxynaphthalene was incubated with the microsomal system with or without the addition of the supernatant fraction, in the absence of SH compounds, the product detected on paper chromatograms was indistinguishable from *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene. The addition of glutathione, cysteine or *N*-acetylcysteine to the system without added supernatant led to the formation of compounds indistinguishable from the glutathione derivative (I), the cysteine derivative (II) and the *N*-acetylcysteine derivative (III) respectively, together with the dihydroxy compound. When the system was incubated with the addition of the supernatant fraction, there was a large increase in the amount of the glutathione derivative (I) formed, whereas the amounts of the cysteine (II) and *N*-acetylcysteine derivative (III) formed were not increased. There was no increase in the yield of the glutathione derivative, compared with that in the first experiment, when supernatant which had been boiled and cooled was added. When 1:2-dihydronaphthalene and microsomes were incubated in the absence of TPN and a reducing system (G 6-P with G 6-P dehydrogenase) no *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene was formed, and the additions of glutathione or cysteine to this system led only to the formation of the products (I) or (II) respectively in amounts comparable with those found in the controls.

The incubation of 1:2-epoxy-1:2:3:4-tetrahydronaphthalene with glutathione in the presence of (*a*) the complete microsome system, (*b*) the system without the supernatant fraction, (*c*) the supernatant fraction alone or (*d*) the boiled supernatant fraction, showed that with either the complete system or with the supernatant fraction alone there was a marked increase in the amounts of the glutathione derivative (I) formed as compared with the amount in the reaction of the epoxide with glutathione in 0·1 M-phosphate buffer, pH 7·4. With the microsomal system without supernatant or with boiled supernatant there were no such increases. The reaction with cysteine or *N*-acetylcysteine in place of glutathione was unaffected by the presence or absence of any part of the system. In all these reactions, large amounts of *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene were formed whether the glutathione derivative was produced or not.

DISCUSSION

The metabolism of 1:2-dihydronaphthalene by rat-liver slices closely resembles that of naphthalene (Booth *et al.* 1960) in that both dihydroxy compounds and glutathione derivatives are formed. The identification of the glutathione derivative formed from 1:2-dihydronaphthalene as *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I) follows from a comparison of its properties on paper chromatograms with those of the synthetic material and from an examination of its acid-hydrolysis products. Additional evidence is provided by the fact that, in whole animals, the mercapturic acid (III) has been shown to be a metabolite of 1:2-dihydronaphthalene (Boyland & Sims, 1960). This mercapturic acid is related to the glu-

tathione derivative (I) in the same way that the mercapturic acid, *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, formed by animals treated with naphthalene, is related to *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione formed from naphthalene by liver slices. The glutathione derivative (I) from 1:2-dihydronaphthalene resembles that formed from naphthalene in that it is not hydrolysed by rat-liver slices or homogenates, whereas it is rapidly converted into the cysteine derivative (II) by rat-kidney homogenates. In the metabolism of 1:2-dihydronaphthalene by rat-kidney slices in the presence of glutathione, the glutathione derivative was not detected, presumably because it was rapidly converted into the mercapturic acid (III).

The formation of 2-naphthol from 1:2-dihydronaphthalene by rat-liver slices is difficult to explain. Possibly there is a hydroxylation at carbon atom 2, followed by dehydrogenation of the product to 2-naphthol. This dehydrogenation is not unexpected since 1:2-dihydronaphthalene itself is apparently dehydrogenated to naphthalene, which is then converted by slices into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione. Moreover, it has been shown by Ayengar, Hayaishi, Nakajima & Tomida (1959) that there is an enzyme present in rabbit liver capable of converting *trans*-1:2-dihydro-1:2-dihydroxynaphthalene into the catechol (1:2-dihydroxynaphthalene). The postulated initial product, 1:2-dihydro-2-hydroxynaphthalene, is easily converted into naphthalene by acid (Bamberger & Lodter, 1895) and could be the source of the naphthalene precursor detected in the urine of animals treated with 1:2-dihydronaphthalene (Pohl & Rawicz, 1919; Boyland & Solomon, 1955).

The results of the action of the microsomal system on 1:2-dihydronaphthalene suggest that an intermediate is formed, which, in the absence of glutathione, cysteine or *N*-acetylcysteine, reacts with water to form *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, whereas in the presence of these SH compounds the derivatives (I), (II) or (III) are also formed. The formation of the intermediate, as detected by its reaction with water or SH compounds to form these products, is dependent on the presence of reduced triphosphopyridine nucleotide. The most likely structure for the intermediate is 1:2-epoxy-1:2:3:4-tetrahydronaphthalene, which has been shown to react with water, glutathione, cysteine and *N*-acetylcysteine at pH 7·4 to give products indistinguishable from those formed in the microsomal system from 1:2-dihydronaphthalene.

The results obtained when the supernatant fraction was added to the system suggest that the supernatant contains an enzyme capable of causing glutathione, but not cysteine or *N*-acetylcysteine, to react with the epoxide. More evidence for this was provided by the experiments in which the epoxide was used: only systems containing supernatant caused an increase in the amount of the glutathione derivative (I) formed, and there was no increase in the amounts of the cysteine and *N*-acetylcysteine derivatives (I and II). Support for the epoxide hypothesis also comes from experiments *in vivo* (Boyland & Sims, 1960), where it was shown that 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene yield the same products when administered to rabbits. Moreover, both compounds yielded only one of the two possible stereoisomeric forms of the mercapturic acid (III), which provides additional evidence that the original reaction of glutathione with the epoxy derivative is enzymic. On the other hand, some evidence suggests that the opening of the oxiran ring with water to form *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene is non-enzymic since, in experiments *in vivo*, both optically active forms of the dihydroxy compound have been obtained (either as the free compounds or as conjugates with glucuronic acid) as metabolites of both 1:2-dihydronaphthalene and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene (Boyland & Sims, 1960). It is uncertain, however, whether or not the isomers are present in equal amounts.

In many respects the metabolism of 1:2-dihydronaphthalene and naphthalene are closely related, both *in vitro* and *in vivo*. Both form mixtures of the optically active forms of the *trans*-dihydroxy compounds and both form substituted cysteine derivatives of closely related structures. This suggests that naphthalene is also converted in the body into a hypothetical epoxide which then reacts either with water (to form *trans*-1:2-dihydro-1:2-dihydroxynaphthalene) or with glutathione [to form *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione]. It has been shown (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth & Boyland, 1958) that a rat-liver microsomal system will convert naphthalene into *trans*-1:2-dihydro-1:2-dihydroxynaphthalene (and into 1-naphthol). The microsomal system with the supernatant fraction converts naphthalene in the presence of glutathione into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione but not into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine in the presence of cysteine (J. Booth, unpublished observations). Thus the results suggest that the enzyme responsible for the reaction between glutathione and epoxide or other intermediate is specific for glutathione but not for the other substrate. One difference in the metabolism of naphthalene and 1:2-dihydronaphthalene is that, with naphthalene, the presence of the supernatant is essential for the formation of the glutathione derivative. If, indeed, naphthalene is

first converted into 1:2-dihydro-1:2-epoxynaphthalene, then this difference may be due to the relative reactivities of this epoxide and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene.

Instances of the formation of epoxides *in vivo* from compounds containing isolated double bonds have been reported previously. Davidow & Radomski (1953), for example, found that the insecticide, Heptachlor, was converted into the epoxide by dogs and rats and the epoxylation of certain steroid double bonds by micro-organisms is also known (Shull & Bloom, 1958). These epoxides are, however, unusually stable.

SUMMARY

1. The preparation of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I) is described.

2. 1:2-Dihydronaphthalene was converted by rat-liver slices into the glutathione derivative (I), which, with rat-kidney homogenate, yielded *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (II) and with rat-kidney slices yielded *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (III).

3. *trans*-1:2-Dihydroxy-1:2:3:4-tetrahydronaphthalene and 2-naphthol were also formed by the action of rat-liver slices on 1:2-dihydronaphthalene.

4. A rat-liver microsomal system converted 1:2-dihydronaphthalene into *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, and, in the presence of glutathione, cysteine or *N*-acetylcysteine, to the respective *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl) derivatives (I), (II) or (III). The formation of the glutathione derivative (I), but not of the derivatives (II) and (III), was increased by the addition of supernatant from the preparation of the microsomes.

5. The addition of glutathione to 1:2-epoxy-1:2:3:4-tetrahydronaphthalene was increased in the presence of the supernatant fraction of rat liver, whereas the addition of cysteine and *N*-acetylcysteine to the epoxide was not affected.

6. The supernatant fraction of a rat-liver homogenate in 0·1 M-phosphate buffer, pH 7·4, appears to contain an enzyme which activates glutathione.

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REFERENCES

Ayengar, P. K., Hayaishi, O., Nakajima, M. & Tomida, I. (1959). *Biochim. biophys. Acta*, **33**, 111.
Bamberger, E. & Lodter, W. (1895). *Liebigs Ann.* **288**, 100.
Booth, J. & Boyland, E. (1958). *Biochem. J.* **70**, 681.
Booth, J., Boyland, E. & Sims, P. (1960). *Biochem. J.* **74**, 117.
Boyland, E. & Sims, P. (1958). *Biochem. J.* **68**, 440.
Boyland, E. & Sims, P. (1960). *Biochem. J.* **77**, 175.
Boyland, E. & Solomon, J. B. (1955). *Biochem. J.* **59**, 518.
Davidow, B. & Radomski, J. L. (1953). *J. Pharmacol.* **107**, 259.
Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.
Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
Pohl, J. & Rawicz, M. (1919). *Hoppe-Seyl. Z.* **104**, 95.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Shull, G. M. & Bloom, B. M. (1958). *U.S. Patent*, no. 2830935.
Sims, P. (1959). *Biochem. J.* **73**, 389.
Straus, F. & Rohrbacher, A. (1921). *Ber. dtsch. chem. Ges.* **54**, 40.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). In *Manometric Techniques and Tissue Metabolism*, 2nd ed. p. 119. Minneapolis: Burgess Publishing Co.

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Biochemical Studies with Methanesulphonyl Esters, Especially β-Chloroethyl Methanesulphonate

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Methanesulphonyl esters are alkylating agents (Ross, 1953), and are used as anti-neoplastic agents. Haddow & Timmis (1951, 1953) reported that several $\alpha\omega$-dimethanesulphonoxy glycols inhibited the growth of the Walker 256 rat carcinoma, 1:4-dimethanesulphonoxy-*n*-butane (Myleran, busulphan) and 1:5-dimethanesulphonoxy-*n*-pentane being most active. Elson (1955, 1957, 1958*a*, *b*)

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and Elson, Galton & Till (1958) have shown that these compounds inhibit cell division in rat bone-marrow. Doses may be given that selectively depress the neutrophil count in the blood. Again, as in the Walker-tumour-inhibition test, busulphan and 1:5-dimethanesulphonoxy-*n*-pentane show the highest activity in depressing the neutrophil count. Physicochemical experiments that may explain their higher activity compared with other members of the series have been reported by Hudson, Timmis & Marshall (1958).

Busulphan has become an accepted palliative agent for treating chronic myeloid leukaemia (Galton, 1956). 1:4-Dimethanesulphonoxy-1:4-dimethyl-*n*-butane (dimethyl busulphan) is chemically more reactive than busulphan, and acts more rapidly than it in depressing the number of circulating neutrophils (Elson, 1957), but is only a weak inhibitor of growth of Walker carcinoma (Hudson & Timmis, 1955; Timmis & Hudson, 1958).

1:6-Dimethanesulphonoxymannitol (mannitol busulphan) is a very soluble, non-toxic compound that inhibits the growth of the Walker carcinoma more than bone-marrow growth when administered to rats (Haddow, Timmis, Leese, Lister & Brown (1957); Haddow, Timmis & Brown (1958); Vargha & Kuszmann, 1959). Clinical trials with this compound have not yet been reported in detail. Haddow & Ross (1956) report that ethyl methanesulphonate (CB 1528) and β-chloroethyl methanesulphonate (CB 1506) inhibit growth of Walker carcinoma. Other experimental anti-neoplastic studies with β-chloroethyl methanesulphonate have been reported by Consoli, Ricciardi, Talarico & Altucci (1957*a*, *b*), although clinical trials with this compound have been disappointing (Papac, Galton, Till & Wiltshaw, 1958).

Roberts & Warwick (1958) have shown that a substantial part of a dose of ethyl methanesulphonate administered to rats appears as *S*-ethyl-*N*-acetyl-L-cysteine and derivatives in the urine. They have also demonstrated (Roberts & Warwick, 1959) that rats probably metabolize most of a dose of busulphan via a cyclic tetrahydrothiophenium ion, showing that *in vivo* some thiol groups are alkylated by methanesulphonyl esters.

There are few reports on the effects of methanesulphonyl esters upon the activities of enzymes containing essential thiol groups. Holzer (1956) reported that 0·8 mM-busulphan caused 50% inhibition of crystalline rabbit-muscle triose-phosphate dehydrogenase *in vitro*.

Ebina & Kurosu (1958) studied the effects of 1:9-dimethanesulphonoxynonane ('nonane') on the respiration and glycolysis of three ascites tumours *in vitro*. Respiration was inhibited, anaerobic glycolysis unaffected and aerobic glycolysis stimulated by this compound.

The work reported here represents an attempt to increase knowledge and understanding of the biological effects of methanesulphonyl esters.

EXPERIMENTAL

Methods

Ehrlich ascites-tumour cells were grown intraperitoneally in inbred black C. 57 mice. Tumour fluid was diluted with approx. 10 vol. of 0·95% NaCl and the cells were separated by low-speed centrifuging. The tumour cells were separated from ascitic plasma and erythrocyte contamination by centrifuging (approx. 200 *g* for 1½ min.) from four or five successive samples of 0·95% NaCl, and were suspended in a Ringer solution for use. Dry-weight determinations were by (*a*) packed-cell volume, estimated in Wintrobe tubes, centrifuged for 54 000 *g*-min. from Krebs–Ringer phosphate (Umbreit, Burris & Stauffer, 1949); 186 mg. dry wt./ml. of packed cells; or (*b*) by weighing a dried pellet of cells in a tared centrifuge tube.

In manometry, Krebs–Ringer phosphate, and Krebs–Ringer bicarbonate (Umbreit *et al.* 1949) were used for respiration and anaerobic glycolysis measurements respectively, with standard techniques in the Warburg apparatus, at 37·5°. Metabolic quotients were calculated from tangents to reaction progress curves, or between the time intervals stated.

Spectrophotometric experiments were carried out at room temperature, 20–22°, in silica cuvettes of 1 cm. light path, in a Unicam SP 500 spectrophotometer. Hydrogen-ion concentrations were measured with the glass electrode.

Materials

Unless otherwise stated, all chemicals were of A.R. quality. Sodium pyruvate was prepared from fractionally redistilled pyruvic acid at 0°, care being taken to prevent the solution becoming alkaline during neutralization. Other chemicals were: L-alanine, L-aspartate, thiamine pyrophosphate from Roche Products Ltd.; L-malic acid, oxaloacetic acid, *cis*-aconitic acid and DL-*iso*citric lactone from California Foundation for Biochemical Research; oxidized and reduced diphosphopyridine nucleotide (DPN and DPNH) and cytochrome *c* from Sigma Chemical Co.; thioctic acid from L. Light and Co. Ltd.; α-oxoglutaric acid and DL-malic acid from British Drug Houses Ltd. Enzymes were prepared and assayed as detailed below.

Enzymes

Succinic dehydrogenase. This was assayed manometrically with phenazine methosulphate as the hydrogen acceptor, in a 10 mM-phosphate (pH 7·2) extract of an acetone-dried powder of rat liver (Singer & Kearney, 1957).

Succinoxidase–cytochrome c reductase. The enzyme in a 0·3 M-sucrose homogenate of rat liver was assayed by measuring the reduction of cytochrome *c* spectrophotometrically, at 550 mμ, in the presence of HCN (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

Succinoxidase. The activity of cell-clumps centrifuged from a brei of rat liver prepared in 0·95% NaCl was estimated manometrically.

Fumarase. This enzyme was partly purified from a mitochondrial fraction of rat liver (prepared in 0·25 M-sucrose), according to Massey (1955). Activity was measured by

following the changes in fumarate concentration spectrophotometrically at 240 mμ (Racker, 1950).

L-*Malic dehydrogenase*. This was prepared from an acetone-dried powder of rat-liver mitochondria to the stage of the first calcium phosphate precipitation, and assayed according to Ochoa (1955).

Lactic dehydrogenase. The fraction of a water homogenate of Ehrlich ascites-tumour cells, precipitated between 0·5 and 0·75 saturation with $(NH_4)_2SO_4$ at 0°, was used. The enzyme was also partly purified from rat muscle and assayed according to Kornberg (1955).

Aconitase. Activity in the malic-dehydrogenase concentrate was measured by following the decrease in *cis*-aconitate at 240 mμ (Racker, 1950).

L-*Glutamic dehydrogenase* was assayed spectrophotometrically by measuring DPN reduction, in a 5 mM-phosphate extract (pH 7·2) of an acetone-dried powder of rat-liver mitochondria (Strecker, 1955).

Pyruvic-dismutation system. A rat-liver homogenate prepared in 0·3 M-sucrose containing 2 mM-ethylenediaminetetra-acetate (EDTA), pH 7·0, was centrifuged free of whole cells and nuclei at 0°. Lactic dehydrogenase, cofactors, pyruvate and phosphate or bicarbonate buffer, pH 7·0, were added. Full conditions are given in Table 7.

RESULTS

Effects in vitro *of methanesulphonyl esters on the anaerobic glycolysis of Ehrlich ascites-tumour cells*

Incubation of Ehrlich ascites-tumour cells for 2 hr. at 37·5° with 10 mM-β-chloroethyl methanesulphonate (with or without 10 mM-nicotinamide), 10 mM-1:6-dimethanesulphonoxymannitol or 10mM busulphan (saturated suspension) did not alter the glycolysis rates of these cells, washed free of drug, relative to controls.

Anaerobic glycolysis measured in the presence of large quantities of β-chloroethyl methanesulphonate, and corrected for acid production from drug hydrolysis, was initially stimulated but later fell (Table 1).

Effects in vitro *of methanesulphonyl esters on the respiration of Ehrlich ascites-tumour cells*

The pure hydrolysis products of β-chloroethyl methanesulphonate (5 mM-β-chloroethanol, 5 mM-sodium methanesulphonate) do not affect the respiration of ascites-tumour cells. With all the esters studied, except dimethyl busulphan, less than 1% was hydrolysed in a 2 hr. incubation period, at 37·5°.

β-Chloroethyl methanesulphonate and other methanesulphonyl esters inhibited the respiration of ascites-tumour cells, the degree of inhibition, for a given concentration of drug, depending upon the substrate being oxidized (Tables 2 and 4). Attempts to protect the respiration of ascites-tumour cells against β-chloroethyl methanesulphonate, or to reverse the inhibition of respiration with thioctic acid, nicotinamide or thiamine, were unsuccessful.

L-Cysteine gave complicated results (Fig. 1), which were interpreted to mean that this compound does not protect the respiration of ascites-tumour cells from β-chloroethyl methanesulphonate, but does cause an initial increase in oxygen uptake due to auto-oxidation and an inhibition of cell respiration, probably due to the destruction of a respiratory factor (Slater, 1949).

The inhibition of ascites-cell respiration increases with time only at high concentrations of β-chloroethyl methanesulphonate. Graphs of respiratory inhibition plotted against time are linear when plotted on log log co-ordinates, for a given drug concentration, when ascites cells are oxidizing added succinate or pyruvate (Fig. 2). When the cells are oxidizing endogenous substrates only, β-chloroethyl methanesulphonate inhibits respiration, but this inhibition remains constant or decreases with time.

Graphs of inhibition of respiration of ascites-tumour cells oxidizing succinate or pyruvate,

Table 1. *Anaerobic glycolysis of Ehrlich ascites-tumour cells in the presence of varying quantities of β-chloroethyl methanesulphonate*

Cell suspension was added from the side arms of the manometer vessels at zero time. Results were calculated from tangents to the CO_2-evolution progress curves corrected for acid production from drug hydrolysis and tissue alkylation. Each flask contained Krebs–Ringer bicarbonate solution, pH 7·3, 36 mM-glucose, 2·50 mg. dry wt. of ascites cells in a total volume of 3·3 ml. The gas phase was $N_2 + CO_2$ (95:5) and the temperature was 37·5°. Acid production from 128 mM-β-chloroethyl methanesulphonate, in the presence of boiled cells, was 13 μl. of CO_2/hr.

Time from beginning of experiment (min.)	β-Chloroethyl methanesulphonate, initial concentration (mM)			
	0	43	85	128
	$Q_{O_2}^{N_2}$* values* and (% control)			
5	+41·5 (100%)	+43·4 (105%)	+48·7 (118%)	+55·8 (135%)
60	+41·5 (100%)	+43·5 (105%)	+48·7 (118%)	+23·9 (58%)
120	+41·5 (100%)	+43·4 (105%)	+21·9 (53%)	+17·5 (42%)
180	+36·7 (100%)	+38·7 (106%)	+7·0 (19%)	+5·2 (14%)

* For explanation of symbols see Dixon (1952).

Table 2. *Respiration of Ehrlich ascites-tumour cells incubated with methanesulphonyl esters as shown*

The inhibition of respiration in the presence of the drug and inhibition of respiration of cells washed free of drug are recorded. Warburg manometers contained the added substrates at a concentration of 20 mM, 0·1 ml. of 30% KOH on a filter-paper roll in the centre well and Krebs–Ringer phosphate (pH 7·4) to a total volume of 4 ml. The atmosphere was O_2 and the temperature 37·5°.

Drug	Substrates	Drug present			Cells washed free of drug		
		Dry wt. of cells (mg./vessel)	Q_{O_2} controls	Inhibition (%)	Dry wt. of cells (mg./vessel)	Q_{O_2} controls	Inhibition (%)
1:5-Dimethanesulphonoxy-n-pentane, 20·6 mM, 105 min.	Endogenous	25·0	– 6·1	61	20·7	– 7·0	2†
	Pyruvate	25·0	– 8·5	81	20·7	– 8·8	2†
	Succinate	—	—	—	20·7	– 9·9	5
β-Chloroethyl methanesulphonate (CB 1506), 5 mM, 120 min.	Pyruvate	*Approx. 18	– 9·3	15	13·6	– 9·4	3†
	Oxaloacetate	*Approx. 18	– 12·6	37	13·6	– 12·0	0
	Pyruvate + oxaloacetate	—	—	—	13·6	– 11·6	0
β-Chloroethyl methanesulphonate (CB 1506), 32·3 mM, 60 min.	Endogenous	23·1	– 7·3	31	20·9	– 6·9	0
	Pyruvate	18·8	– 9·1	51	20·9	– 9·3	0
	Succinate	28·1	– 10·7	46	20·9	– 10·9	0
β-Chloroethyl methanesulphonate (CB 1506), 56 mM, 105 min.	Endogenous	23·1	– 6·4	62	13·9	– 6·0	11
	Pyruvate	18·8	– 9·3	85	13·9	– 8·1	7
	Succinate	20·5	– 10·4	70	13·9	– 10·1	12

† Inhibition too small to be significant.

* Average of several determinations.

Fig. 1. Apparent respiration rates of Ehrlich ascites-tumour cells. Each Warburg flask contained 11·2 mg. of cells in 3·3 ml. of Krebs–Ringer phosphate containing 32 mM-sodium pyruvate, with 0·1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well. Additions: △, none; ▲, β-chloroethyl methanesulphonate, 19 mM; □, L-cysteine, 5 mM; ○, L-cysteine, 5 mM, and β-chloroethyl methanesulphonate, 19 mM. Results are calculated from tangents to the oxygen-uptake curves.

Fig. 2. Inhibition of respiration of Ehrlich ascites-tumour cells after different periods in various concentrations of β-chloroethyl methanesulphonate. Each flask contained 18·8 mg. of cells in Krebs–Ringer phosphate, with 25 mM-sodium pyruvate (- - - -) or 20·4 mg. of cells and 20 mM-sodium succinate, (—). Results were calculated from graphs of oxygen uptake. Each vessel contained 0·1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well. Numbers on graphs represent the concn. of β-chloroethyl methanesulphonate as mM.

plotted against the logarithm of β-chloroethyl methanesulphonate concentration, are sigmoid in form. The same results, plotted as probit inhibition against log β-chloroethyl methanesulphonate concentration, produce straight lines at low concentrations of drug, but at higher drug concentrations the graphs curve upward, the upward curvature increasing with time as the degree of respiratory inhibition increases (Fig. 3). The concentration of β-chloroethyl methanesulphonate causing 50% inhibition of respiration decreases linearly with the logarithm of the experimental time. With β-chloroethyl methanesulphonate and 1:5-dimethanesulphonoxy-*n*-pentane, the inhibition of respiration of ascites cells is independent of pH in the range pH 5·0–7·4.

The inhibition of respiration of ascites-tumour cells by low concentrations of β-chloroethyl methanesulphonate is completely reversed by washing the cells free of drug, but with concentrations greater than approx. 30 mM the inhibition of respiration cannot be completely removed. It is above approx. 30 mM-β-chloroethyl methanesulphonate that the respiration of ascites cells is progressively inhibited, when pyruvate or succinate are the exogenous substrates of respiration (Fig. 2).

These facts indicate that β-chloroethyl methanesulphonate, and possibly other methanesulphonyl esters, inhibit the respiration of ascites-tumour cells by a physical effect. Unlike 2:4-dinitrophenol and other compounds that uncouple oxidative phosphorylation, β-chloroethyl methanesulphonate does not prevent glucose depressing the oxygen uptake (Crabtree effect) in ascites-tumour cells (Table 3).

Between 90 and 120 min. from the beginning of the experiment, the respiration *in vitro* of washed ascites cells oxidizing endogenous substrates is slightly inhibited in cells in contact with 5 mM-β-chloroethyl methanesulphonate (2% mean of 19 determinations). The addition of a number of metabolites to suspensions of ascites-tumour cells raises the respiration rate by various amounts. β-Chloroethyl methanesulphonate (5 mM) inhibits the respiration of ascites cells to an extent related

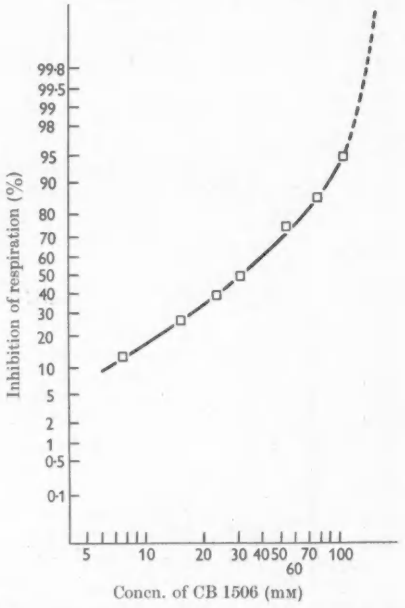

Fig. 3. Inhibition of respiration of Ehrlich ascites-tumour cells by various concentrations of β-chloroethyl methanesulphonate (CB 1506), 120 min. after the addition of drug. Results, calculated from tangents to oxygen-uptake curves, are plotted on probit-log co-ordinates. Each vessel contained 15·3 mg. of cells in 4·0 ml. of Krebs–Ringer phosphate with 25 mM-sodium pyruvate, and 0·1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well.

Table 3. *Respiration of Ehrlich ascites-tumour cells in the presence and absence of glucose, β-chloroethyl methanesulphonate and 2:4-dinitrophenol*

Experiments were carried out in Warburg manometers in an atmosphere of O_2 at 37·5°. Each flask contained Krebs–Ringer phosphate to a final volume of 4·0 ml., 15·8 mg. dry wt. of cells and 0·1 ml. of 30% KOH on a filter-paper roll in the centre well.

Additions					
Glucose, 38 mM	−	−	+	+	+
β-Chloroethyl methanesulphonate (CB 1506), 5 mM	−	+	−	+	−
2:4-Dinitrophenol, 0·1 mM	−	−	−	−	+
Time interval (min.)	$Q_{O_2}^{O_2}$ values				
0–30	−10·8	−10·6	−6·6	−6·6	−9·9
30–60	−9·4	−8·9	−6·0	−6·0	−10·1
60–90	−8·3	−8·1	−5·4	−5·8	−9·0
90–120	−7·7	−7·4	−5·4	−5·4	−9·2

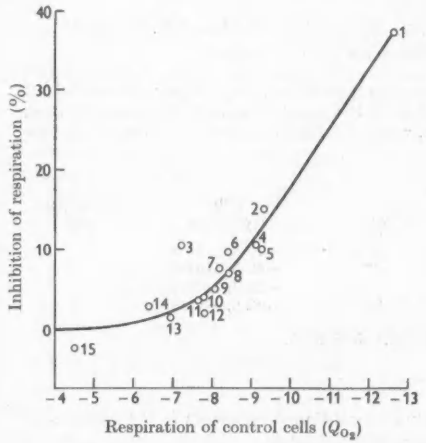

Fig. 4. Inhibition of respiration of Ehrlich ascites-tumour cells by 5 mM-β-chloroethyl methanesulphonate. Each vessel contained an average of 18 mg. of cells in 4·0 ml. of Krebs–Ringer phosphate; added substrates were initially 24 mM, adjusted to pH 7·0 with NaOH, except for oxaloacetate, where modified Ringer with 70 mM-phosphate buffer, pH 7·3, was used. Results are calculated from oxygen-uptake graphs between 90 and 120 min. from the addition of drug. In the centre well of each vessel there was 0·1 ml. of 30% (w/v) KOH on a filter-paper roll. Substrates used (numbers in parentheses show the number of determinations averaged for the point shown): 1, oxaloacetate (3); 2, pyruvate (3); 3, L-alanine (4); 4, succinate (5); 5, DL-lactate (4); 6, L-glutamate (4); 7, L-aspartate (5); 8, citrate (4); 9, fumarate (4); 10, α-oxoglutarate (4); 11, DL-malate (4); 12, *cis*-aconitate (3); 13, endogenous substrates (19); 14, DL-*iso*citrate (2); 15, DL-methionine (2).

Table 4. *Inhibition of respiration of Ehrlich ascites-tumour cells caused by 30 mM-β-chloroethyl methanesulphonate*

Results were calculated between 90 and 120 min. from time the drug was added to cells in Warburg manometers. Each vessel contained Krebs–Ringer phosphate to a final fluid volume of 4·0 ml., an average of about 15 mg. dry wt. of cells and 0·1 ml. of 30% KOH on a filter-paper roll in the centre well. Incubation was at 37·5° in O_2. The tumours were the second or third passage from the tumour bank and were growing slowly. The $Q_{O_2}^{N_2}$ values were below normal.

Substrate	$Q_{O_2}^{N_2}$ (controls)	Inhibition (%)	No. of determinations
Glucose (75 mM)	−3·5	46	(3)
Endogenous	−3·8	26	(4)
DL-Malate (30 mM)	−4·0	21	(1)
Citrate (30 mM)	−5·0	65	(2)
Pyruvate (30 mM)	−7·8	64	(3)
Succinate (30 mM)	−8·9	46	(3)

to the respiration rate (Fig. 4), but 30 mM-β-chloroethyl methanesulphonate does not inhibit respiration to this pattern (Table 4).

Metabolism of ascites-tumour cells from mice previously injected with methanesulphonyl esters

The respiration rates of ascites-tumour cells, oxidizing pyruvate or endogenous substrates, from mice injected intraperitoneally 4–4·5 hr. previously with 5 m-moles (/kg.) of ethyl methanesulphonate, β-chloroethyl methanesulphonate, 1:6-dimethanesulphonoxy mannitol, dimethyl busulphan or busulphan in iso-osmotic sodium chloride, or force-fed with 120 m-moles of busulphan/kg., 24 or 48 hr. before use, do not differ significantly from controls.

Enzyme experiments

Soluble succinic dehydrogenase was not affected by 10 mM-β-chloroethyl methanesulphonate, but was inhibited to a small variable extent by 1:5-dimethanesulphonoxy-*n*-pentane (25 mM), busulphan (2·3 mM), 1:6-dimethanesulphonoxymannitol (37 mM), and a saturated suspension of dimethyl busulphan.

After a preincubation period of 2 hr., β-chloroethyl methanesulphonate inhibited a succinoxidase preparation when an excess of cytochrome *c* was added (Table 5).

A succinoxidase–cytochrome *c*-reductase preparation was inhibited by various methanesulphonyl esters (Table 6). The inhibition caused by β-chloroethyl methanesulphonate was investigated in more detail. Lineweaver & Burk (1934) plots showed 'mixed inhibition' curves (Fig. 5).

Enzymes not inhibited by incubation with 10 mM-β-chloroethyl methanesulphonate for 2 hr. at 37·5° or by various other methanesulphonyl esters were fumarase, L-malic dehydrogenase, lactic dehydrogenase, aconitase and L-glutamic dehydrogenase.

A pyruvic-dismutation system was variably inhibited by methanesulphonyl esters and a high concentration of diethyl ether (Table 7). Essentially identical results were obtained with bicarbonate–carbon dioxide or phosphate buffers.

DISCUSSION

The methanesulphonyl esters are alkylating agents that *in vivo* react with thiol groups (Roberts & Warwick, 1958, 1959). Of the thiol enzymes studied a pyruvic-dismutation system and a succinoxidase–cytochrome *c*-reductase system were inhibited. In both cases it is known that enzymic activity depends on the structural integrity of an enzymic complex, and that lipid solvents or other lipid-destroying agents are effective in disorganizing them.

Table 5. *Succinoxidase activity of a rat-liver brei incubated with and without β-chloroethyl methanesulphonate and cytochrome c for various times*

Succinate, final concn. 10 mM, was added from the side arm at the times shown. Experiments were carried out in Warburg manometers in O_2 at 37·5°. Each vessel contained 10·3 mM-β-chloroethyl methanesulphonate, 1·46 mg. dry wt. of liver, Krebs–Ringer phosphate to a final volume of 4·0 ml. and 0·1 ml. of 30% KOH on a filter-paper roll in the centre well.

Cytochrome *c* (0·05%)	β-Chloroethyl methanesulphonate (CB 1506), 10·3 mM	Q_{O_2} (30 min.)	Inhibition (%)	Q_{O_2} (120 min.)	Inhibition (%)
−	−	−68·9	.	−41·8, −45·9	.
−	+	−65·8	4*	−42·5, −41·8	3*
+	−	−72·3	.	−53·7, −50·7	.
+	+	−65·8	9	−42·5, −41·8	19

* Inhibition too small to be significant.

Table 6. *Succinoxidase–cytochrome c-reductase activity of a rat-liver homogenate in the presence of varying amounts of methanesulphonyl esters*

Rat liver, homogenized in 0·25 M-sucrose + 2 mM-EDTA at pH 7·0 and 0°, was centrifuged free of whole cells and nuclei, diluted with EDTA solution and allowed to stand for 30 min. at 0°. Esters were in contact with the enzyme for 15 min. at room temp., 20°, before assay. Final concentrations: cytochrome *c*, approx. 40 μM; potassium succinate, 2·5 mM, pH 7·0; phosphate buffer, 17 mM, pH 7·0; HCN, 0·3 mM; EDTA, pH 7·0, 0·85 mM. The assay was at room temp., 20°, in 1 cm. silica cuvettes at 550 mμ. Mean control rate of cytochrome *c* reduction, $+\Delta E_{550\,m\mu}$/10 min. was 0·527.

Ester	Concentration (mM)	Inhibition (% of control rate)
Busulphan	0·6	22
1:5-Dimethanesulphonoxy-*n*-pentane	4·0	19
1:6-Dimethanesulphonoxy mannitol	25·0	32
Ethyl methanesulphonate (CB 1528)	25·0	24

Fig. 5. Inhibition of rat-liver succinoxidase–cytochrome *c* reductase by β-chloroethyl methanesulphonate. Lineweaver & Burk (1934) plot of $1/v$ against $1/[S]$, where reaction velocity v is $+\Delta E_{550\,m\mu}$/10 min., $[S]$, succinate concentration (M). Drug was in contact with enzyme for 10 min. at room temp., 22°, before assay. Medium contained mM-EDTA and 16·6 mM-phosphate buffer, pH 7·0. △, Controls; ▲, 10·0 mM-β-chloroethyl methanesulphonate; □, 25·1 mM-β-chloroethyl methanesulphonate.

In vitro, low concentations of methanesulphonyl esters inhibited the respiration, but not the anaerobic glycolysis of ascites-tumour cells. The observed effects may be explained by physical actions of the compounds studied. This concept is supported, for β-chloroethyl methanesulphonate, by the complete reversibility, by washing, of respiratory inhibition with drug concentrations up to approx. 30 mM. At these concentrations, inhibition of respiration does not increase with time (Fig. 2); in fact inhibition seems to be instantaneously established by the presence of the drug.

No explanation is offered for the increased inhibition of respiration of ascites cells at elevated respiration rates, caused by different substrates, but data showing similar trends have been recorded elsewhere; for example, brain slices respiring in the presence of 0·08% of luminal or 3·3 mM-chloretone (Jowett & Quastel, 1937; Quastel, 1955). In some tissues the succinoxidase system is more resistant to drugs and physical damage than the DPNH-oxidase system. This may explain why 30 mM-β-chloroethyl methanesulphonate inhibits the respiration of ascites-tumour cells to a lesser

Table 7. *Pyruvate-dismutation system assayed in the presence and the absence of various methanesulphonyl esters*

Esters were in contact with enzymes for 45 min. at 37·5° before pyruvate was added from the side arm. Results were calculated from CO_2-evolution curves between 10 and 30 min. from tipping. Warburg manometers: total fluid volume, 3·0 ml.; atmosphere, N_2; final concentration of rat liver, 8% (homogenized in 0·3 M-sucrose + 2 mM-EDTA, pH 7·0, freed from whole cells and nuclei by centrifuging and saturated with CO_2); lactic dehydrogenase concentrate 1·0 ml. Final concentrations: sodium pyruvate, 46 mM; DPN, 2 mM; nicotinamide, 0·5 mM; thiamine pyrophosphate, 0·5 mM; $MgCl_2$, 0·5 mM; sodium fumarate, 0·3 mM; sucrose, 0·3 M; EDTA, 2 mM; potassium phosphate buffer, 10 mM, pH 7·0.

Ester	Activity of preparation (+μl. of CO_2/hr.)	Inhibition (%)
None (controls)	+130, +130	0
Busulphan (2·0 mM)	+120, +127	5
1:5-Dimethanesulphonoxy-*n*-pentane (10 mM)	+131, +124	2*
1:6-Dimethanesulphonoxy mannitol (10 mM)	+126, +126	3*
β-Chloroethyl methanesulphonate (CB 1506) (10 mM)	+112, +112	14
Diethyl ether (approx. 120 mM)	+101, +102	22

* Inhibition too small to be significant.

extent when they are oxidizing succinate than when they are oxidizing pyruvate or citrate. The progressive irreversible inhibition of ascites-tumour-cell respiration by high concentrations of β-chloroethyl methanesulphonate might be due to the disruption of a progressively greater proportion of the cells present.

SUMMARY

1. The respiration of Ehrlich ascites-tumour cells is inhibited by a variety of methanesulphonyl esters *in vitro*. This inhibition is reversed by washing the cells free of drug, reversal being complete at low drug concentrations.

2. Kinetic evidence and enzyme studies are presented which support the hypothesis that inhibition of respiration by β-chloroethyl methanesulphonate is due to physical effects of this ester on the hydrogen-transport chain of the cell.

This paper constitutes a partial report of results submitted to the University of London as a Ph.D. thesis. I am grateful to Professor E. Boyland for his interest in this work. Dr J. Booth kindly supplied rat-liver mitochondria, and generous gifts of methanesulphonyl esters were made by Mr G. M. Timmis, Dr W. Davis, Dr W. C. J. Ross and Dr S. S. Brown. A Royal Marsden Hospital Gordon Jacobs Research Fellowship was held throughout this work, which was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Consoli, G., Ricciardi, S., Talarico, L. & Altucci, P. (1957*a*) *Boll. Soc. ital. Biol. sper.* **33**, 682.

Consoli, G., Ricciardi, S., Talarico, L. & Altucci, P. (1957*b*) *Boll. Soc. ital. Biol. sper.* **33**, 685.

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.

Dixon, M. (1952). *Manometric Methods*, 3rd ed. Cambridge University Press.

Ebina, T. & Kurosu, M. (1958). *J. nat. Cancer Inst.* **20**, 1023.

Elson, L. A. (1955). *Brit. J. Haematol.* **1**, 104.

Elson, L. A. (1957). *Advances in Radiobiology*, p. 372. Edinburgh: Oliver and Boyd Ltd.

Elson, L. A. (1958*a*). *Ann. N.Y. Acad. Sci.* **68**, 826.

Elson, L. A. (1958*b*). *Biochem. Pharmacol.* **1**, 39.

Elson, L. A., Galton, D. A. G. & Till, M. (1958). *Brit. J. Haematol.* **4**, 355.

Galton, D. A. G. (1956). *Advanc. Cancer Res.* **4**, 73.

Haddow, A. & Ross, W. C. J. (1956). *Nature, Lond.*, **177**, 995.

Haddow, A. & Timmis, G. M. (1951). *Acta Un. int. Cancr.* **7**, 469.

Haddow, A. & Timmis, G. M. (1953). *Lancet*, i, 207.

Haddow, A., Timmis, G. M. & Brown, S. S. (1958). *Nature, Lond.*, **182**, 1164.

Haddow, A., Timmis, G. M., Leese, C. L., Lister, J. H. & Brown, S. S. (1957). *Rep. Brit. Emp. Cancer Campgn*, no. 35, p. 41.

Holzer, H. (1956). *Die Medizinische*, **15**, 576.

Hudson, F. R. & Timmis, G. M. (1955). *Rep. Brit. Emp. Cancer Campgn*, no. 33, p. 5.

Hudson, R. F., Timmis, G. M. & Marshall, R. D. (1958). *Biochem. Pharmacol.* **1**, 48.

Jowett, M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 565.

Kornberg, A. (1955). In *Methods in Enzymology*, vol. 1, p. 441. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Lineweaver, H. C. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Massey, V. (1955). In *Methods in Enzymology*, vol. 1, p. 729. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Papac, R., Galton, D. A. G., Till, M. & Wiltshaw, E. (1958). *Ann. N.Y. Acad. Sci.* **68**, 1126.

Quastel, J. H. (1955). In *Origins of Resistance to Toxic Agents*, p. 209. Ed. by Sevag, M. G., Reid, R. D. & Reynolds, O. E. New York: Academic Press Inc.
Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.
Roberts, J. J. & Warwick, G. P. (1958). *Biochem. Pharmacol.* **1**, 60.
Roberts, J. J. & Warwick, G. P. (1959). *Biochem. J.* **72**, 3P.
Ross, W. C. J. (1953). *Advanc. Cancer Res.* **1**, 397.
Singer, T. P. & Kearney, E. B. (1957). *Meth. biochem. Anal.* **4**, 73.
Slater, E. C. (1949). *Biochem. J.* **45**, 14.
Strecker, H. J. (1955). In *Methods in Enzymology*, vol. 2, p. 220. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Timmis, G. M. & Hudson, R. F. (1958). *Ann. N.Y. Acad. Sci.* **68**, 727.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.
Vargha, L. & Kuszmann, J. (1959). *Naturwissenschaften*, **46**, 84.

Biochem. J. (1960) **77**, 194

Preparation of Crystalline *Pseudomonas* Cytochrome *c*-551 and its General Properties

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A simple method has already been devised for extensive purification of four respiratory components from *Pseudomonas aeruginosa* in a true water-soluble state without the aid of detergents (Horio, 1958*a*). These components were called *Pseudomonas* (*P*)-cytochrome-554, *P*-cytochrome-551, *P*-blue protein and *P*-cytochrome oxidase (Horio, 1958*b*; Horio, Higashi, Matsubara *et al.* 1958). The same micro-organism also contains another cytochrome, *P*-cytochrome-560, which is similar to the so-called cytochrome b_1 and which has not yet been solubilized without the aid of sodium cholate (Horio, 1958*a*). From studies on purified respiratory components, cellular fragments and whole cells, Horio (1958*b*) and Yamanaka (1959) have concluded that in the electron-transferring system of *P. aeruginosa* *P*-cytochrome-551 and *P*-blue protein should be functional at a site corresponding to cytochrome *c* of animal mitochondria. Preliminary studies on chemical and physical properties of the purified components showed that *P*-cytochrome-551 bears a closer resemblance to a typical cytochrome *c* than it does to *P*-blue protein (Horio, 1958*a*, *b*).

The first crystallization of cytochrome *c* was accomplished by Bodo (1955), who isolated the pigment from king penguin (*Aptenodytes patagonica*). It was extracted by the method of Keilin & Hartree (1937, 1945) and further purified with the use of the ion-exchange resin Amberlite IRC-50 (Margoliash, 1954*a*, *b*). By very similar methods, crystalline cytochrome *c* has since been obtained from a variety of sources, such as baker's yeast (Hagihara, Horio, Yamashita, Nozaki & Okunuki, 1956), ox heart (Kuby, Paléus, Paul & Theorell, 1956), horse heart (Nozaki, Yamanaka, Horio & Okunuki, 1957), fish heart (Hagihara *et al.* 1957) and wheat germ (Hagihara, Tagawa, Morikawa, Shin & Okunuki, 1958). In all cases, cytochrome *c* was purified from the crude to the purest state by chromatography on a weak cationic ion-exchange resin by procedures established by Paléus & Neilands (1950) and by Margoliash (1954*a*, *b*).

Despite the many similarities of *P*-cytochrome-551 to other types of cytochrome *c*, the standard procedure for the purification of the types of cytochrome *c* is inefficient for the purification of *P*-cytochrome-551 (Horio, 1958*a*, *b*). The main reason appears to be the acidic isoelectric point of *P*-cytochrome-551, which decreases the adsorptive power of a weak cationic resin such as Amberlite IRC-50 within its effective pH range.

In this paper, a suitable method for the preparation of crystalline *P*-cytochrome-551 from *P. aeruginosa* is presented together with a description of its general properties. A preliminary report on this work has been given by Horio, Higashi, Nakai, Kusai & Okunuki (1958).

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MATERIALS AND METHODS

Organism. A strain of *P. aeruginosa* was used which produces large amounts of cytochromes when it is grown as submerged cultures.

Media. Two types of media were employed: bouillon–peptone medium and nitrate medium. The former was composed of 5 g. of Ehrlich meat extract (Kyokuto Pharmaceutical Ind. Co. Ltd., Tokyo), 10 g. of Polypepton (Daigo Nutritional Chemicals Co. Ltd., Osaka), 2·5 g. of NaCl and 1 l. of water. The pH was adjusted to 7·0 with 5 N-NaOH. The nitrate medium was composed of 1 kg. of Ehrlich meat extract, 1 kg. of Polypepton, 2 kg. of KNO_3, 0·64 kg. of KH_2PO_4, 0·36 kg. of Na_2HPO_4 and 100 l. of tap water (pH 6·6).

Growth of the organism. The organism was maintained on the bouillon–peptone medium solidified with agar (2%, w/v). Bulk growth of the organism for the purpose of *P*-cytochrome-551 purification was carried out in three stages. The bouillon–peptone medium (15 ml.) was inoculated and incubated at 37° for 24 hr. with vigorous shaking. The resulting culture was added to 6 l. of bouillon–peptone medium and incubated at 37° for 24 hr. Finally, this bouillon–peptone culture was added to 90 l. of the nitrate medium in a deep steel tank and incubated at 37° without shaking until the pH of the medium rose to approx. 8 (usually 20 hr.). During the incubation a Kotaki photoelectric colorimeter, type 5-D, was used with the filter no. 11 (610–750 mμ) to estimate turbidity of the medium. The extinction began to increase when the pH rose above 7 (approx. 9 hr. after the inoculation) and became maximal when the pH reached approx. 8. Prolonged incubation, which leads to a further rise in pH, should be avoided since the cells may then autolyse and liberate the cytochromes into the culture medium. At the end of incubation the medium usually contained approx. 8 mg. (wet wt.) of the organisms/ml.

Preparation of resin. The small beads of Duolite CS-101 (polymethacrylic acid resin, supplied by Chemical Process Co., Redwoods City, Calif., U.S.A.) were ground and the resin powder from 150 to 200 mesh was collected. The mesh size of the resin is not critical, but the size selected combines ease in pretreating the resin with a high adsorbing capacity. The resin was heated with N-NaOH at approx. 80° for 15–20 min. and then washed completely with water. The alkaline treatment and washing were repeated. The resulting resin was suspended in N-HCl, stirred for 15–20 min. and washed with water until the pH of the resin suspension rose to about 5. The product will be called the H-form resin. The H-form resin was further suspended in 0·1 M-ammonium phosphate buffer, pH 6·0, and then washed with water. This treatment with buffer was repeated until the pH of the resin suspension became 6·0. This resin will be called the pH 6 resin. Amberlite CG-50 can be used in place of Duolite CS-101, although its efficiency is slightly lower.

Preparation of activated aluminium oxide. From activated aluminium oxide for chromatography (Takeda Pharmaceutical Co. Ltd., Osaka, Japan), a powder from 200 to 300 mesh was collected and treated with alkali and acid in the same way as the resin. After a complete washing with water, the aluminium oxide was immersed in 0·1 M-ammonium phosphate buffer, pH 6·0, and then washed with water. This treatment with buffer and the subsequent washing were repeated until the pH of the suspension became 6·0. The product will be called the pH 6 aluminium oxide.

Absorption spectra. A Shimadzu photoelectric spectrophotometer, type QB-50, with silica cuvettes (1 cm. optical path) was used. The purity of *P*-cytochrome-551 during fractionation is expressed as the ratio $(E^{red.}_{551} - E^{red.}_{570})/E_{280}$, where $E^{red.}_{551}$ and $E^{red.}_{570}$ are extinctions of the dithionite-reduced sample at 551 and 570 mμ respectively, and E_{280} is the extinction at 280 mμ of a sample not treated with reducing or oxidizing agents. The absorption spectra of crystalline *P*-cytochrome-551 were measured with a Cary recording spectrophotometer, model 14.

Zone electrophoresis on a vertical starch column. Zone electrophoresis was carried out on a vertical column (30cm. × 3cm. diam.) packed with raw-potato-starch powder, under the following experimental conditions: 0·1 M-sodium phosphate buffer, pH 7·0; approx. 20 mA at 350–400 V with anode at the bottom of the column; temp., 4–5°. The starch (Wako Pure Chemical Industries, Co. Ltd., Osaka, Japan) was washed three times with a large amount of N-HCl, then sufficiently with water. The powder was washed with a large amount of buffer, and only the buffered powder able to sediment on standing within half an hour was used.

Oxidation–reduction potentials. The method of Henderson & Rawlinson (1956) for measuring E'_0 of heart-muscle cytochrome *c* was used. The solution of crystalline *P*-cytochrome-551 was dialysed against 0·2 M-sodium phosphate buffer, pH 6·50, for 3 days in a refrigerator. The dialysed sample was reduced by addition of a suitable amount of sodium dithionite just before assay. The reduced sample was assayed by titration with 3 mM-potassium ferricyanide dissolved in the phosphate buffer. The concentration of *P*-cytochrome-551 ($E^{red.}_{551}$ 0·66, 3·00 ml.) was previously adjusted so that the assay could be accomplished with 15–20 drops (0·15–0·20 ml.) of the ferricyanide solution.

Electrophoretic analysis. A Tiselius apparatus was used. The cells contained equilibrated buffer (0·03 M-sodium phosphate buffer or sodium acetate buffer, adjusted with NaCl so that I was 0·10) as the overlying solution. A sample of *P*-cytochrome-551 recrystallized three times, was dialysed at 4–5° against the buffer for 3 days. Runs to examine the homogeneity of *P*-cytochrome-551 were carried out in an ice–water bath, and runs to calculate electrophoretic mobilities in a water bath regulated at 20±0·05°. Calculations were based upon the ascending boundaries.

Ultracentrifugal analysis. Sedimentation measurements were made with a Spinco model E ultracentrifuge (rotor type AN-A). The runs were carried out at 259 700 *g* at room temperature (approx. 20°). The average of initial and final rotor temperatures was used for calculating $S_{20,w}$ by the method of Svedberg & Pedersen (1940).

Diffusion analysis. Diffusion was studied by the cylindrical-lens schlieren method with the use of cells of the Neurath type. The temperature of the thermostat was regulated within ±0·01° according to the technique of Moriguchi (1932) and measurements were carried out at 20°. $D_{20,w}$ was calculated by the inflexion-point method (Neurath, 1942).

Partial specific volume. The densities of the solution and of the solvent were measured at 20±0·05° with an Ostwald-type pycnometer (3 ml. capacity).

Analysis of iron and nitrogen. A sample of *P*-cytochrome-551 was dialysed with stirring against glass-distilled water

for 72 hr. at 4–5° with several changes of the outside water. The dialysed solution was dried at 60° over P_2O_5 until the residue attained constant weight. Iron was estimated by the *o*-phenanthroline method (Fortune & Mellon, 1938; Thiel, Heinrich & Hengel, 1938) after the residue had been subjected to the wet oxidation described by Sandell (1950). Nitrogen was assayed by a micro-Kjeldahl method.

EXPERIMENTAL AND RESULTS

Purification of P-*cytochrome*-551

Step 1. *Acetone-dried powder of cells.* The cells grown in approx. 100 l. of nitrate medium were collected by filtration on a Büchner funnel containing filter paper and a layer of Celite (Hyflo Super-cel). About 760 g. (wet wt.) of cells was collected. The cells and Celite were mixed with 5 l. of solid CO_2–acetone in a large, wide-mouth Thermos flask. The temperature was maintained below 5° during this acetone treatment. Five minutes were required to complete the mixing. The mixture was allowed to stand for 10 min., rapidly filtered on a Büchner funnel and the collected cells were rapidly washed with 2 l. of cold acetone on the same funnel. The resulting powder was dried overnight at room temp. (10–20°) in a current of air.

Step 2. *Extraction of respiratory components from the acetone-dried cells.* The acetone-dried cells with Celite were well mixed with 10 l. of 0·1 M-sodium citrate adjusted to pH 7·6 with N-NaOH. The mixture was heated at 40° for 10 min. with continuous stirring and was filtered at room temperature through a layer of Celite on a Büchner funnel. The filtration took 1 day and yielded 8·3 l. of dark-brown filtrate, pH 6·2. A second extraction was performed with 10 l. of the sodium citrate either with or without heat treatment. The second extract contained one-tenth of the amount of *P*-cytochrome-551 of the first extract, and the purity of the second extract was much lower than that of the first extract.

Step 3. *Treatment of the extract with rivanol and precipitation of the respiratory components by ammonium sulphate.* To 8·3 l. of the first filtrate in step 2, 1 l. of aqueous 4 % rivanol (2:5-diamino-7-ethoxyacridinium lactate monohydrate, supplied by Fujisawa Pharmaceutical Ind. Co. Ltd., Osaka) was added. On mixing, a bulky fibrous precipitate was produced which was removed by filtration through gauze. To the filtrate was added 9·6 kg. of ammonium sulphate (95 % saturation). After being kept overnight in a refrigerator, the resulting precipitate was collected on a Büchner funnel containing Celite. The precipitate plus Celite was dialysed against running tap water for 3 days in a cold room, with some changes of dialysis tubing. During this dialysis, brown impurities were precipitated. The volume of the dialysed and filtered solution was approx. 1·3 l.

Step 4. *Treatment of the dialysed solution with* pH 6 *resin.* The dialysed solution was passed through a column (50 cm. × 3 cm. diam.) packed with pH 6 resin. The rivanol, not removed by dialysis in step 3, was adsorbed at the top of the column. Just below the yellow zone of the adsorbed rivanol was a second zone consisting of all the *P*-cytochrome oxidase, some of the *P*-cytochrome-554 and a large amount of impurities. The *P*-cytochrome-551 together with *P*-blue protein passed through the column. The *P*-cytochrome oxidase was subsequently removed from the column by elution with M-ammonium phosphate buffer, pH 7·0, and stored for further purification (Horio, Higashi, Matsubara *et al.* 1958).

Step 5. *Adsorption of* P-*cytochrome*-551 *on H-form resin.* The solution which passed through the pH 6 resin in step 4 was passed through a column (50 cm. × 3 cm. diam.) packed with H-form resin. The *P*-cytochrome-551 together with *P*-blue protein and *P*-cytochrome-554 were completely adsorbed. The charged column was washed with water and then eluted with M-ammonium phosphate buffer, pH 7·0. The eluate was kept neutralized with frequent addition of dilute sodium hydroxide. The part of the eluate which showed an absorption band around 550 mμ (156 ml.) was collected. Up to this step, the content of *P*-cytochrome-551 could not be precisely assayed spectrophotometrically because of coloured impurities. In one case the final yield of cytochrome was calculated on the basis of the eluate at this stage containing 100 arbitrary units (Table 1).

Step 6. *Chromatography of* P-*cytochrome*-551 *on* pH 6 *aluminium oxide.* The reddish brown eluate from the H-form resin was dialysed against running tap water in a cold room for 1 day and then passed through a column (20 cm. × 3·5 cm. diam.) of pH 6 aluminium oxide. Brown impurities were adsorbed at the top of the column and *P*-blue protein and *P*-cytochrome-551 were adsorbed just below the brown band. *P*-blue protein has a higher affinity for the aluminium oxide than *P*-cytochrome-551. However, in most cases it was difficult to observe it, since it was partly in the colourless reduced form and the oxidized form was largely masked by brown impurities and the red colour of *P*-cytochrome-551. The brown, top portion of the aluminium oxide was scraped away and the rest of the column was washed with water. (All attempts to fractionate the adsorbed substances by means of eluting buffers before removal of the brown impurities led to a serious decrease in yield.) The adsorbed *P*-cytochrome-551 slowly moved down the column during washing. When a sample of eluate, treated with dithionite and examined with a microspectroscope, showed an absorption band around 550 mμ the washing was stopped. The

Table 1. *Summary of purification of* Pseudomonas *cytochrome-551*

Main steps	Volume (ml.)	Purity ($E^{red.}_{551} - E^{red.}_{570}/E_{280}$)	Yield (%)
1. Extract of acetone-dried powder of cells	8300	—	—
2. Eluate from H-form Duolite CS-101 column	156	0·028	(100)
3. Eluate from pH 6-aluminium oxide column	200	0·090	59
4. Fractionation by ammonium sulphate (60–90% saturation)	30	0·12	52
5. Freeze-dried powder	(708 mg.)	0·14	52
6. Zone-electrophoresis			
(*a*) First run	5	0·82	46
(*b*) Second run	5	1·00	41
7. Crystallization			
(*a*) Cryst. ppt.	2·9	1·18	28
(*b*) First crystals	2·5	1·26	22
(*c*) Second crystals	1·7	1·27	15
(*d*) Third crystals	1·2	1·27	9

washed column was then eluted with M-ammonium phosphate buffer, pH 7·0, and the fractions containing *P*-cytochrome-551 were collected (200 ml.). This eluate, which also contained *P*-blue protein, was brownish red. If it was reduced by sodium dithionite, its colour changed to pink, characteristic of reduced *P*-cytochrome-551, since the *P*-blue protein present in the eluate was simultaneously bleached by the reducing agent. The eluate also contained a trace of *P*-cytochrome-554.

Step 7. *Fractionation of* P*-cytochrome-551 with ammonium sulphate.* To 200 ml. of the step 6 eluate, 76 g. of ammonium sulphate was added (approx. 60% saturation), and the mixture was centrifuged at 10 000 *g* for 20 min. The brown precipitate was discarded, the brownish red supernatant was treated with a further 56 g. of ammonium sulphate (90% saturation) and centrifuged again. The supernatant was yellowish green and contained *P*-blue protein and *P*-cytochrome-554 but very little *P*-cytochrome-551. The precipitate contained *P*-cytochrome-551 and *P*-blue protein but virtually no *P*-cytochrome-554. The *P*-blue protein in the supernatant fluid was precipitated by saturation with ammonium sulphate.

Step 8. *Freeze-drying and zone electrophoresis of* P*-cytochrome-551.* The sample which was precipitated between 60 and 90% saturation of ammonium sulphate in step 7 was dissolved in a small amount of water, dialysed for 3 days in a cold room and freeze-dried. The resulting powder (708 mg.) could be stored at 4–5° for more than 3 months. It was dissolved in 4 ml. of 0·1 M-sodium phosphate buffer, pH 7·0, and a small amount of insoluble material was removed by centrifuging. The clarified supernatant was placed in the middle of a vertical column packed with starch and electrophoresis was

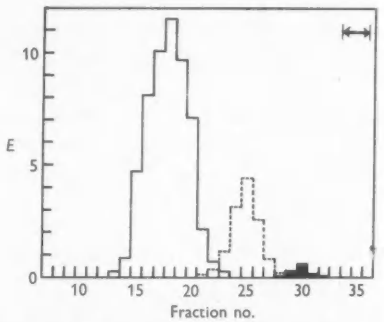

Fig. 1. Fractionation of *Pseudomonas* cytochrome-551 by zone electrophoresis on a starch column. The eluate was collected as 2 ml. fractions. If the sample were to be eluted from the position where it was originally placed, it would appear in the fractions shown by the arrow. $E^{ox.}_{630}$ was measured on a portion of the eluate fractions after oxidation with excess of potassium ferricyanide. The other extinctions were measured with portions of the eluate fractions previously reduced by excess of sodium dithionite. Detection of *P*-cytochrome-551, *P*-cytochrome-554 and *P*-blue protein contained in the eluate fractions was carried out microspectroscopically and their approximate quantities are expressed by $E^{red.}_{551} - E^{red.}_{560}$, $E^{red.}_{554} - E^{red.}_{564}$ and $E^{ox.}_{630} - E^{red.}_{630}$ respectively. ——, *P*-cytochrome-551; - - -, *P*-blue protein; ▬, *P*-cytochrome-554.

carried out under the conditions described above. Brown impurities migrated downwards fairly rapidly, whereas *P*-blue protein and *P*-cytochrome-551 migrated very slowly in the same direction. When the electrophoresis was left running overnight, *P*-blue protein and *P*-cytochrome-551 became separately observable as blue and red zones

respectively. But electrophoresis for 3 days was necessary to complete the separation and to force most of the brown impurities out of the bottom of the column. At this stage, the column was eluted with 0·1 M-sodium phosphate buffer, pH 7·0, with the aid of a 2 ml.-fraction collector. First of all, brown impurities at the bottom of the column were eluted and these were followed by *P*-cytochrome-551 as reddish pink fractions and *P*-blue protein, in its reduced form, as colourless fractions (Fig. 1). Although some of the *P*-cytochrome-554 had been adsorbed on the pH 6 resin, a little remained to be eluted after *P*-blue protein. All fractions that followed *P*-cytochrome-554 showed negligible extinction at 280 mμ. Those *P*-cytochrome-551 fractions whose $E^{red.}_{551}/E_{280}$ ratios were higher than 0·65 were combined and subjected to a second electrophoresis under the same experimental conditions as the first run. Fractions with $E^{red.}_{551}/E_{280}$ ratio greater than 0·9 were collected in a second run. When they were combined the ratio was 1·00, indicating that the purity of *P*-cytochrome-551 had risen approximately seven times as a result of electrophoresis (Table 1). Whenever the ratio was higher than 0·5–0·6, *P*-cytochrome-551 could be easily crystallized by the following procedure. However, in order to get the best yield of purest material, it was found advantageous to use samples purified by the second run. Fractions containing *P*-blue protein were nearly saturated with ammonium sulphate and stored in a refrigerator, together with the *P*-blue protein precipitated in step 7, for further study.

Step 9. *Precipitation of* P-*cytochrome*-551 *based upon differences in solubility of its oxidized and reduced forms.* The *P*-cytochrome-551 collected in step 8 was completely oxidized by addition of a minimal amount of potassium ferricyanide, nearly saturated with ammonium sulphate, and centrifuged down to a compact pellet. This was dissolved in a volume of water, such that the value of $E^{551}_{1\,cm.}$ after reduction with dithionite was $\leqslant 30$. If the concentration is too low, the yield of *P*-cytochrome-551 in the following procedure is greatly decreased. During all crystallization procedures the pH was adjusted to approx. 7·5 with ammonia. To the oxidized solution, finely powdered ammonium sulphate was added in small portions until a slight turbidity appeared (30–40 % saturation). The concentration of ammonium sulphate required to produce slight turbidity varied, and was especially affected by temperature and protein concentration. The turbid solution was immediately centrifuged at the same or a slightly lower temperature. The precipitation of a small portion of the *P*-cytochrome-551 at this stage indicates that the concentration of ammonium sulphate is appropriate for the following procedure. The deep-red supernatant was reduced by the addition of a small amount of sodium dithionite, when it became lighter in colour and also very turbid. The turbid solution was allowed to stand overnight and then centrifuged. The precipitate consisted of fine-needle crystals of *P*-cytochrome-551, in a yield of about 70 % from the mother solution. The brownish red supernatant contained the rest of the cytochrome and a small amount of *P*-blue protein. To the crystalline precipitate of *P*-cytochrome-551, water was added dropwise and with gentle stirring until the suspension exhibited a silken sheen. The crystals were again collected in a centrifuge. The purity of the *P*-cytochrome-551 at this stage was usually higher than 93 %. The crystals were dissolved in a little water and reduced with a small amount of dithionite ($E^{551}_{1\,cm.} \leqslant 30$). The solution was clarified by centrifuging and recrystallized as before by addition of ammonium sulphate. Needle crystals appeared in the turbid solution after storage for a few hours at or just below room temperature (Plate 1). After three recrystallizations the iron content was 0·69 % and the ratio, $E^{red.}_{551} - E^{red.}_{570}/E_{280}$ was 1·27, and these values were unchanged by further recrystallizations. Allowing a solution of suitable turbidity to stand at a temperature much below that at which the turbidity has been obtained should be avoided, since the solubility of *P*-cytochrome-551 in ammonium sulphate solution increases as the temperature is lowered. The final product represents a 45-fold purification of the eluate from H-form resin (step 2), and a 1500-fold purification of the extract of acetone-dried cells if it is assumed that all of the *P*-cytochrome-551 contained in this extract was collected on the column of H-form resin.

The *P*-cytochrome-551 could also be easily crystallized in its oxidized form if, after the oxidation with ferricyanide, the solution was treated with ammonium sulphate until it became fairly turbid and then allowed to stand for a few days. Oxidized *P*-cytochrome-551 crystallized both as needles and as octahedra: the yield, however, was lower than when the pigment was crystallized in its reduced form.

General properties of P-*cytochrome*-551

Iron and nitrogen content. A three-times recrystallized sample of *P*-cytochrome-551 contained 0·69 ± 0·005 % (3) of iron and 16·2 ± 0·03 % (3) of nitrogen. Figures in parentheses indicate the number of estimations. On the basis of its iron content, the minimum molecular weight of *P*-cytochrome-551 is 8100.

Absorption spectra. *P*-cytochrome-551, recrystallized three times, was dissolved in 0·1 M-sodium phosphate buffer, pH 7·0, and completely oxidized by the addition of a slight excess of potassium ferricyanide. The oxidized solution was dialysed against

Crystals of *Pseudomonas* cytochrome-551 (reduced form) from a solution in ammonium sulphate (×800).

the same buffer for 3 days in a cold room with several changes of buffer. This solution was used to obtain the oxidized absorption spectrum in Fig. 2, and the same solution, after treatment with sodium borohydride, gave the reduced spectrum. On the basis of iron content the molar extinction coefficient of the reduced pigment at 551 mμ is $28{\cdot}3 \times 10^6$ mole^{-1} cm.^{2}. The absorption maxima of *P*-cytochrome-551 are located at wavelengths significantly different from those of crystalline cytochrome *c* from ox-heart muscle. The ratios of extinctions at the absorption maxima of the two cytochromes also show small but definite differences (Table 2).

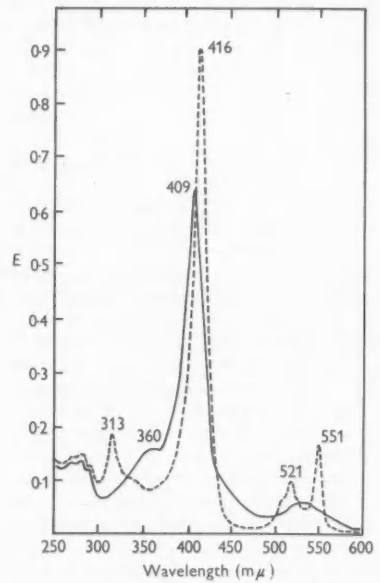

Fig. 2. Absorption spectra of crystalline *Pseudomonas* cytochrome-551. The sample was recrystallized three times and its absorption spectrum was measured in 0·1 M-sodium phosphate buffer, pH 7·00, at 23°. The cytochrome was reduced by addition of a small amount of sodium borohydride. ——, Oxidized form; - - - - -, reduced form.

Ultracentrifugal and diffusion analyses. *P*-cytochrome-551, recrystallized three times, was dialysed against 0·02 M-sodium phosphate buffer, pH 7·0 (I 0·10, adjusted with NaCl), at 4–5° for 72 hr. Ultracentrifugal analyses of solution containing 0·4–1·1 % (w/v) of protein showed that the sample consisted of a homogeneous protein. $S_{20,w}$ was calculated to be $1{\cdot}34 \pm 0{\cdot}07$ s (4) and was nearly independent of concentration. Diffusion analyses were carried out with the same solutions of *P*-cytochrome-551. The value of $D_{20,w}$ $14{\cdot}8 \pm 0{\cdot}9 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ (4), was also nearly independent of concentration.

Partial specific volume. A sample recrystallized three times gave the value $\bar{v}$ 0·710 ml. g.$^{-1}$.

Molecular weight. The molecular weight of *P*-cytochrome-551 was calculated to be 7600 from the equation,

$$M = \frac{RTS}{(1-\bar{v}\rho)D} = 2{\cdot}44 \times 10^{10} \times \frac{S_{20,w}}{(1-0{\cdot}9982\,\bar{v})D_{20,w}}$$

(Carter, 1941; Lauffer & Stanley, 1940). This value is close to the minimum molecular weight based upon iron content (8100), which indicates that this cytochrome possesses one iron atom per molecule. Unlike the haem of haemoglobin, the haem of *P*-cytochrome-551 is not split from the protein moiety by treatment with acetone–HCl. The haem–protein linkage is thus of the *c*-type.

Table 2. *Absorption spectra of crystalline* Pseudomonas *cytochrome*-551 *and ox-heart-muscle cytochrome* c

P-cytochrome-551		Ox-heart-muscle cytochrome *c*	
Absorption max. (mμ) Reduced	$E/E^{red.}_{551}$	Absorption max. (mμ) Reduced	$E/E^{red.}_{550}$
551	(1·0)	550*	1·0)
521	0·58	520	0·60
416	5·3	415	4·7
313	1·1	313	1·3
290	—	290	—
280	—	280	—
Oxidized		Oxidized	
530	0·38	530	0·40
409	3·6	408	3·7
360	0·92	358	1·0
290	0·59	290	0·65
280	0·74	280	0·82

* Molar extinction at 550 mμ of crystalline ox-heart-muscle cytochrome *c* was $28{\cdot}2 \times 10^6$ mole^{-1} cm.

Electrophoretic analysis. A sample of *P*-cytochrome-551, recrystallized three times, was dialysed at 4–5° against 0·03 M-sodium phosphate buffer or sodium acetate buffer (I 0·10, adjusted with NaCl). Tests for homogeneity were carried out at 0° and at two pH values, 5·75 and 8·13. In all experiments the current was 10 mA. Both the descending and ascending schlieren patterns revealed the presence of a single protein. For calculation of electrophoretic mobility, runs were carried out at 20° with the results shown in Fig. 3. The current was 5·0 mA. In sodium phosphate buffer, pH 7·0 (I 0·10), the cytochrome migrated with a uniform descending boundary and had a mobility of $4{\cdot}35 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$. The isoelectric point of *P*-cytochrome-551 was found to be 4·70.

Normal oxidation–reduction potential. E'_0 of *P*-cytochrome-551 was found to be $+0{\cdot}286 \pm 0{\cdot}004$ v (3) at pH 6·50 and 20°.

DISCUSSION

The first successful water-solubilization and partial purification of bacterial *c*-type cytochrome was obtained with *Rhodospirillum rubrum* (Vernon, 1953). Vernon & Kamen (1954) reported that this cytochrome has the following physicochemical properties: absorption maxima, 550 mμ, 521 mμ, and 415 mμ in its reduced form; 530–535 mμ and 408 mμ in its oxidized form; mol.wt., 16 000; E'_0, 0·338 v at pH 7·0. Kamen & Takeda (1956) purified a bacterial cytochrome *c* from *P. aeruginosa*, which showed absorption maxima in its reduced form at 552, 520 and 416 mμ, and in its oxidized form at 530 and 408–410 mμ. A *c*-type cytochrome obtained in a pure form from *P. aeruginosa* by Horio (1958*a*) showed in its reduced state an absorption maximum at 550·5 mμ, which differs slightly but definitely from the wavelength given by Kamen & Takeda (1956). Besides this cytochrome, Horio (1958*a*, *b*) found another cytochrome easily extractable from the same micro-organism, and, after considerable purification, the latter, in its reduced form, showed an absorption maximum at 554 mμ. These two bacterial cytochromes were tentatively called *P*-cytochrome-551 and *P*-cytochrome-554, according to the nomenclature of Scarisbrick (1947). At present it seems likely that the cytochrome *c* purified by Kamen & Takeda (1956) could be a mixture of *P*-cytochrome-551 and *P*-cytochrome-554.

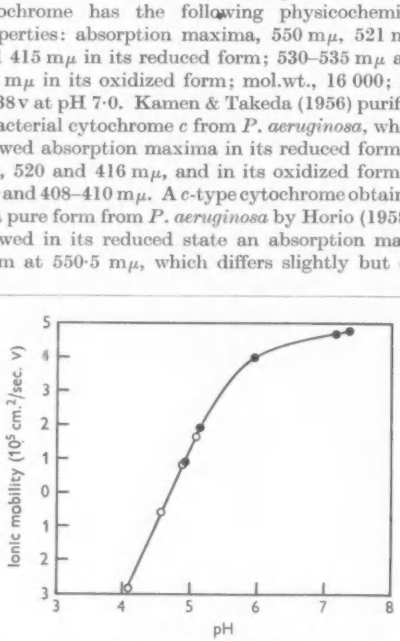

Fig. 3. Ionic mobility of crystalline *Pseudomonas* cytochrome-551. ○, Acetate buffer; ●, phosphate buffer; I 0·10; temperature 20°.

Unlike *P*-cytochrome-551, typical cytochrome *c* compounds can be easily purified by column chromatography on a weak cationic resin such as Amberlite IRC-50. For example, the oxidized form of cytochrome *c* extracted from baker's yeast can be separated into four main fractions. All of them show the same absorption spectrum in their reduced as well as in their oxidized forms, and it could be inferred that this yeast contains at least four different types of cytochrome *c*. Comparative studies of types of cytochrome *c* extractable from baker's yeast, heart muscle of ox and horse and breast muscle of pigeon have shown that it is a common occurrence for several *c*-type pigments to appear during chromatographic purification. However, it can now be accepted that there is only one native cytochrome *c* in each living organism and that all additional chromatographic species or 'modified cytochrome *c*' result from certain changes in the coiling of the protein structure (Nozaki *et al.* 1957; Nozaki, Mizushima, Horio & Okunuki, 1958; Mizushima, Nozaki, Horio & Okunuki, 1958; Yamanaka, Mizushima, Nozaki, Horio & Okunuki, 1959). Nozaki (1960) has found that ox-heart cytochrome *c*, when modified by acids, exists in a dimeric form within a neutral pH range. Cytochrome *c* compounds which have been partially digested by proteolytic enzymes fail to be adsorbed on weakly acid resins, and they show absorption spectra notably different from those of the native and modified cytochrome *c* (Nozaki, Mizushima, Yamanaka, Horio & Okunuki, 1959). Since crystallizability is a common property of native cytochrome *c*, we believe that crystalline *P*-cytochrome-551 is the native form of the pigment.

Egami *et al.* (1957) proposed a system of nomenclature for cytochromes based upon the nature of the haem group, on the species of organism and upon the oxidation–reduction potential. According to their system, *P*-cytochrome-551 would be called 'cytochrome *c* (*Pseudomonas aeruginosa*, E'_0 0·286 v)'. We propose this as the formal name, and '*Pseudomonas* cytochrome *c*-551' as the common name, for the pigment described in this paper.

SUMMARY

1. *Pseudomonas* cytochrome *c*-551 has been isolated and crystallized from *P. aeruginosa*, which was grown in submerged culture with nitrate as a terminal electron acceptor. The purification involves collection by H-form Duolite CS-101, chromatography on activated aluminium oxide, zone electrophoresis on a starch column and precipitation followed by crystallization from ammonium sulphate solution.

2. After three recrystallizations, *Pseudomonas* cytochrome *c*-551 was a homogeneous protein according to electrophoretic and ultra-centrifugal criteria.

3. The cytochrome shows absorption maxima at 551, 521, 416, 316, 290 and 280 mμ in the reduced form, and at 530, 409, 360, 290 and 280 mμ in the oxidized form. The molar extinction coefficient at 551 mμ (α-absorption maximum) is $28{\cdot}3 \times 10^6$ mole^{-1} cm.^{2} at pH 7·00.

4. The cytochrome contains 16·2% of nitrogen and 0·69% of iron. The latter figure indicates a minimum molecular weight of 8100.

5. Values of sedimentation constant, diffusion constant and partial specific volume at pH 7·0 (I 0·10) are $S_{20,w}$, $1{\cdot}34 \times 10^{-13}$ cm. sec.$^{-1}$; $D_{20,w}$, $14{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$; $\bar{v}$, 0·710. The molecular weight calculated from these values is 7600 and the cytochrome thus contains one iron atom per molecule.

6. The isoelectric point of the cytochrome is 4·70, and E_0' (at pH 6·50 and at 20°) is +0·286 v.

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REFERENCES

Bodo, G. (1955). *Nature, Lond.*, **176**, 829.

Carter, R. O. (1941). *J. Amer. chem. Soc.* **63**, 1960.

Egami, F., Ishimoto, M., Mori, T., Ogura, Y., Okunuki, K. & Sato, R. (1957). *J. Biochem., Tokyo*, **44**, 619.

Fortune, W. B. & Mellon, M. G. (1938). *Industr. Engng Chem. (Anal.)*, **10**, 60.

Hagihara, B., Horio, T., Yamashita, J., Nozaki, M. & Okunuki, K. (1956). *Nature, Lond.*, **178**, 629.

Hagihara, B., Morikawa, I., Tagawa, K., Yamashita, J., Nozaki, M. & Okunuki, K. (1957). *Nature, Lond.*, **179**, 249.

Hagihara, B., Tagawa, K., Morikawa, I., Shin, M. & Okunuki, K. (1958). *Nature, Lond.*, **181**, 1950.

Henderson, R. W. & Rawlinson, W. A. (1956). *Biochem. J.* **62**, 21.

Horio, T. (1958*a*). *J. Biochem., Tokyo*, **45**, 195.

Horio, T. (1958*b*). *J. Biochem., Tokyo*, **45**, 267.

Horio, T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M. & Okunuki, K. (1958). *Biochim. biophys. Acta*, **29**, 297.

Horio, T., Higashi, T., Nakai, M., Kusai, K. & Okunuki, K. (1958). *Nature, Lond.*, **182**, 1307.

Kamen, M. D. & Takeda, Y. (1956). *Biochim. biophys. Acta*, **21**, 518.

Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc.* B, **122**, 298.

Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.

Kuby, S. A., Paléus, S., Paul, K. G. & Theorell, H. (1956). *Acta chem. scand.* **10**, 148.

Lauffer, M. A. & Stanley, W. M. (1940). *J. biol. Chem.* **135**, 463.

Margoliash, E. (1954*a*). *Biochem. J.* **56**, 527.

Margoliash, E. (1954*b*). *Biochem. J.* **56**, 535.

Mizushima, H., Nozaki, M., Horio, T. & Okunuki, K. (1958). *J. Biochem., Tokyo*, **45**, 845.

Moriguchi, N. (1932). *J. chem. Soc. Japan*, **53**, 381.

Neurath, H. (1942). *Chem. Rev.* **30**, 357.

Nozaki, M. (1960). *J. Biochem., Tokyo*, **47**, 592.

Nozaki, M., Mizushima, H., Horio, T. & Okunuki, K. (1958). *J. Biochem., Tokyo*, **45**, 815.

Nozaki, M., Mizushima, H., Yamanaka, T., Horio, T. & Okunuki, K. (1959). *Symp. enz. Chem.* **13**, 31.

Nozaki, M., Yamanaka, T., Horio, T. & Okunuki, K. (1957). *J. Biochem., Tokyo*, **44**, 453.

Paléus, S. & Neilands, J. B. (1950). *Acta chem. scand.* **4**, 1024.

Sandell, E. B. (1950). *Colorimetric Determination of Traces of Metals*. New York: Interscience Publishers Inc.

Scarisbrick, R. (1947). *Rep. Progr. Chem.* **44**, 226.

Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. New York: Oxford University Press.

Thiel, A., Heinrich, H. & Hengel, E. (1938). *J. Amer. chem. Soc.* **71**, 756.

Vernon, L. P. (1953). *Arch. Biochem. Biophys.* **43**, 492.

Vernon, L. P. & Kamen, M. D. (1954). *J. biol. Chem.* **211**, 643.

Yamanaka, T. (1959). *J. Biochem., Tokyo*, **46**, 1289.

Yamanaka, T., Mizushima, H., Nozaki, M., Horio, T. & Okunuki, K. (1959). *J. Biochem., Tokyo*, **46**, 121.

Biochem. J. (1960) **77**, 202

Micro-Determination of Soluble Silica in Urine

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Silicon may be determined in solution by the spectrophotometric method of Dienert & Wandenbulche (1923), as modified by King & Stantial (1933). This involves the formation of the yellow silicomolybdate and the reduction of this complex to molybdenum blue by 1-amino-2-naphthol-4-sulphonic acid reagent. Measurement of this colour provides a means of determining silicon.

A disadvantage of this method of determining silicon is that phosphate interferes by forming phosphomolybdate, which is similarly reduced to molybdenum blue. Various methods have been used for preventing phosphate interference during silicon determination. Khaler (1941) used mineral acids to prevent the formation of phosphomolybdate. Complex-forming agents such as oxalic acid (Schwartz, 1942; De Sesa & Rogers, 1954) or citric acid (Case, 1944; De Sesa & Rogers, 1954) were used to bleach the interfering phosphomolybdate while leaving the silicomolybdate unaffected. Paul & Pover (1960*a*) have developed a method of selectively removing phosphomolybdate by ethyl acetate. In their experiments, silicon was determined in the aqueous phase after reduction of the silicomolybdate to molybdenum blue.

These methods of eliminating phosphate during the determination of silicon are successful when only small amounts of phosphate are present to interfere. However, in the presence of high concentrations of phosphate, such as are found in rat and human urines, these methods are not applicable unless the urines are diluted considerably. This is undesirable, for when silica is present in trace quantities it might escape determination.

When phosphate is present in high concentrations, it can be removed by precipitation. Thayer (1930) claimed that phosphate was completely removed by precipitation with calcium chloride and ammonia at about pH 8·0 without simultaneous removal of any silicate. King & Stantial (1933) also reported precipitation of phosphate by calcium chloride and ammonia during silica determination in urine. However, none of these workers investigated the effect of pH on silicate and phosphate precipitation. As it has been the author's experience that calcium chloride and ammonia cause precipitation of silicate at pH 8·0, a systematic study of the effect of pH on silicate and phosphate precipitation by calcium chloride and ammonia was made. From the results obtained it was possible to develop an accurate and reproducible method of determining silicon in rat and human urines.

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EXPERIMENTAL

Materials

When possible, reagents of A.R. grade were used.

Silica standard. Solutions of soluble silica were prepared by fusing 10·0 mg. of finely divided quartz with 0·5 g. of powdered sucrose and 1·5 g. of sodium peroxide in a Parr bomb, and dissolving the fused material in 100 ml. of water in a polythene beaker. Further solutions of silicate were prepared by dilution and used as standards.

Phosphate standard. A stock solution of phosphate was prepared by dissolving 0·5 g. of anhydrous disodium hydrogen phosphate in water and diluting to 100 ml. Standard solutions were prepared from it by dilution.

Ammonium molybdate. The tetrahydrate (5·0 g.) was dissolved in 100 ml. of water.

1-Amino-2-naphthol-4-sulphonic acid reagent. The acid (200 mg.) was dissolved in water with 12·0 g. of sodium metabisulphite and 2·4 g. of sodium sulphite heptahydrate and diluted to 100 ml. After the solution had stood overnight it was filtered into a brown-glass bottle and kept in a cool place.

Calcium chloride. The anhydrous salt (10·0 g.) was dissolved in 100 ml. of water.

Ammonia solution. Aqueous ammonia (sp.gr. 0·880) was diluted to give a 2N-solution, which was stored in a polythene bottle. This solution contained a negligible amount of silicate.

Urine samples. Rat and human urines were collected and stored in polythene beakers containing liquid paraffin.

Trimethylhexadecylsilane. This was synthesized by the method of Paul & Pover (1960*b*). The method involved the methylation of trichlorohexadecylsilane (Found: Si 9·39. $(CH_3)_3SiC_{16}H_{33}$ requires Si, 9·40%; the infrared spectrum of trimethylhexadecylsilane showed two bands at 8·025 and 11·8–12·02 μ).

Methods

Precipitation of phosphate in urine. Urine (2·0 ml.) was mixed with 8·0 ml. of water. A portion (4·0 ml.) of the diluted urine was acidified by adding 0·2 ml. of acetic acid

and the mixture was shaken. Calcium chloride solution (4·0 ml. of 10%, w/v) was added, together with 2·0 ml. of aqueous ammonia (2N). The mixture was then shaken and set aside for 15 min. This solution, which was at pH 7·0, was filtered and the filtrate used for the colorimetric determination. Blanks containing water in place of urine, brought to pH 7·0, were similarly prepared.

Determination of silicon. A portion (2·0 ml.) of the filtrate was pipetted into a 50 ml. separating funnel, followed by the addition, with shaking, of 2·0 ml. of N-hydrochloric acid. Ammonium molybdate solution (4·0 ml. of 5%, w/v) was then added and the solution mixed by shaking for 3 min. This solution had a pH of 1·0. It was then extracted once with 20 ml. of ethyl acetate for 30 sec. The aqueous phase was separated and 4·0 ml. of 10% (w/v) citric acid solution added to it with shaking. The solution was allowed to stand for 2 min., after which 1·0 ml. of the amino-naphtholsulphonic acid reagent was added with shaking, and the resulting solution set aside for 10 min. to allow maximum colour to develop. Any precipitate formed was removed at this stage by centrifuging. The extinction of the blue solution, which was linear to silicon concentration in the range 5–60 μg. of SiO_2, was then measured against a similarly treated blank at 690 mμ.

Effect of pH *on the precipitation of phosphate and silicate.* To each of several 4 ml. samples of disodium hydrogen phosphate solution (2·26 mg./ml.) or sodium silicate (0·125 mg. of SiO_2/ml.) were added 4·0 ml. of 10% calcium chloride solution and 2·0 ml. of 2N-ammonia solution. The mixtures were brought to different pH values in the range 4·0–10·0, by adding acetic acid. After centrifuging, the phosphate, or silicate, was determined in the supernatant.

Determination of phosphate. Phosphate was determined by the method of King (1932).

RESULTS AND DISCUSSION

The results recorded in Table 1 demonstrate that the present method of determining silicon is accurate and reproducible. King & Stantial (1933) reported that when 1-amino-2-naphthol-4-sulphonic acid reagent was used as the reducing agent a persistent 'blank' colour was obtained. The author has found that the addition of citric acid before the reduction eliminated this 'blank' colour without affecting the reduction by the aminonaptholsulphonic acid reagent.

In Table 2 are recorded results demonstrating the effect of pH on phosphate precipitation. These results were obtained by determining the remaining phosphate concentration in the supernatant, after precipitation and centrifuging. At pH 4·0 no precipitation of phosphate occurred; at pH 5·0, 35·5% of the phosphate was precipitated. At pH 7·2, only 5·4% of the total phosphate was present in the supernatant, 94·6% having been precipitated. Total precipitation of phosphate was obtained between pH 9·0 and 10·0.

If the results of phosphate precipitation are compared with those of Table 3 it can be seen that, unlike phosphate, silicate is not precipitated

Table 1. *Recovery of known amounts of silicon*

Samples of trimethylhexadecylsilane were fused in a Parr bomb and their silicon contents determined by the modified colorimetric method with 1-amino-2-naphthol-4-sulphonic acid reagent in the presence of citric acid, to effect reduction of silicomolybdate (for details see Experimental section).

Wt. of trimethylhexadecylsilane fused (mg.)	Silicon present (mg.)	Silicon found (mg.)	Error (%)
15·6	1·46	1·50	2·7
15·0	1·42	1·40	1·4
16·0	1·50	1·45	3·3
14·9	1·40	1·40	0·0
15·7	1·47	1·47	0·0
14·6	1·37	1·41	2·9
28·7	2·71	2·68	1·1
15·1	1·42	1·36	4·2

Mean 1·95 ± 0·38

Table 2. *Effect of* pH *on phosphate precipitation*

Solutions of sodium phosphate, each containing 11·30 mg. of anhydrous disodium hydrogen phosphate, were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values below. After centrifuging, the clear supernatant was examined for phosphate (for details see Experimental section).

pH	Amount of phosphate recovered in supernatant (mg.)	Phosphate recovered in supernatant (%)
4·0	11·30	100·0
5·0	7·29	64·5
6·0	1·16	10·3
7·2	0·605	5·4
8·2	0·467	4·1
9·0	0·275	2·4
10·0	<0·005	<0·05

Table 3. *Effect of* pH *on silicate precipitation*

Solutions of sodium silicate, each containing 0·500 mg. of SiO_2, were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values given below. After centrifuging, the clear supernatant was examined for silicate.

pH	Amount of silicate recovered in supernatant (mg. of SiO_2)	Silicate recovered in supernatant (%)
5·0	0·500	100·0
6·0	0·500	100·0
7·0	0·500	100·0
8·0	0·445	89·0
9·0	0·407	81·4
10·0	0·330	66·0

between pH 5·0 and 7·0. However, silicate precipitation occurred at pH 8·0, 10% of the total silicate being precipitated at this pH. Nevertheless, even at pH 10·0, at which pH all the phosphate was precipitated, only 34% of the silicate was precipitated.

In order to determine whether co-precipitation of silicate occurred during phosphate precipitation, the effect of pH on silicate precipitation in the presence of phosphate was studied. These results reported in Table 4 are in good agreement with those of Table 3, thus demonstrating that co-precipitation of silicate does not occur during phosphate precipitation.

By taking advantage of the difference at pH 7·0 between the precipitation of phosphate and silicate, a method of determining silicate in urine was developed. As only a small amount of phosphate escapes precipitation at pH 7·0, whereas silicate is not precipitated at this pH, it was possible to precipitate the bulk of the phosphate at neutral pH and then eliminate the remaining phosphate by extracting the phosphomolybdate with ethyl acetate in the manner described by Paul & Pover (1960*a*). When this technique was applied, accurate and reproducible determinations of small amounts of silicon in rat and human urine were obtained (Table 5). As rat and human urines contain large amounts of phosphate (about 30 mg./ml. in rat urine), the method should be generally useful for determining trace amounts of silicon in very high concentrations of phosphate. In the experiments reported here, silica, in the presence of up to about 100 times its own concentration of phosphate, was accurately determined.

Table 4. *Effect of* pH *on silicate precipitation in the presence of phosphate*

Solutions of sodium silicate, each containing 0·228 mg. of silica plus 25·0 mg. of disodium hydrogen phosphate, were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values given below. After centrifuging, the clear supernatant was examined for silicate.

pH	Amount of silicate recovered in supernatant (mg. of SiO_2)	Silicate recovered in supernatant (%)
5·0	0·228	100·0
6·0	0·228	100·0
7·0	0·228	100·0
8·0	0·210	92·1
9·0	0·189	82·9
10·0	0·156	68·4

SUMMARY

1. An accurate and reproducible method of determining small amounts of silica in rat and human urine is described. The method involves precipitating the bulk of the phosphate present in urine, by calcium chloride and ammonia at pH 7·0, and removing the remaining phosphate by selective extraction of the phosphomolybdate with ethyl acetate. Silica was then determined in the aqueous phase after reduction of the silicomolybdate by 1-amino-2-naphthol-4-sulphonic acid reagent in the presence of citric acid.

2. The method is capable of general applicability to determinations of trace amounts of silica in high concentrations of phosphate.

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Table 5. *Recovery of silica in rat and human urines*

Urine (2 ml.) was made up to 10 ml. with water. A portion (4 ml.) of this solution was then treated with the precipitating reagents. After centrifuging, soluble silica was determined in the supernatant. (For details see text.) To 2 ml. of the same urine, 2 ml. of silicate solution was added and the whole made up to 10 ml. After precipitation and centrifuging as described above, the total silica content of the urine was determined. All values are expressed as milligrams.

Urine	SiO_2/2 ml. of urine	SiO_2 added to 2 ml. of urine	Total SiO_2 found in 2 ml. of urine	SiO_2 recovered
Rat	0·110	0·260	0·370	0·260
	0·150	0·300	0·450	0·290
	0·110	0·400	0·510	0·390
	0·140	0·100	0·240	0·09
	0·120	0·07	0·190	0·07
Human	0·020	0·240	0·260	0·250
	0·021	0·400	0·421	0·400
	0·030	0·300	0·330	0·300
	0·020	0·340	0·360	0·330
	0·020	0·100	0·120	0·100

REFERENCES

Case, O. P. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 309.
De Sesa, M. A. & Rogers, L. B. (1954). *Analyt. Chem.* **26**, 1278.
Dienert, F. & Wandenbulche, F. (1923). *C.R. Acad. Sci., Paris*, **176**, 1478.
Khaler, H. L. (1941). *Industr. Engng Chem. (Anal.)*, **13**, 536.
King, E. J. (1932). *Biochem. J.* **26**, 292.
King, E. J. & Stantial, H. (1933). *Biochem. J.* **27**, 990.
Paul, J. & Pover, W. F. R. (1960*a*). *Anal. chim. acta*, **22**, 185.
Paul, J. & Pover, W. F. R. (1960*b*). *Arch. Biochem. Biophys.* **87**, 312.
Schwartz, M. C. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 893.
Thayer, L. A. (1930). *Industr. Engng Chem. (Anal.)*, **2**, 277.

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Amino Acid-Activating Enzymes in Muscle

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The enzymic 'activation' of amino acids (Hoagland, 1955) is recognized and measured by the formation of hydroxamic acids in the presence of amino acids, adenosine triphosphate and hydroxylamine, or by the incorporation of radioactive pyrophosphate into adenosine triphosphate in the presence of amino acids. There is ample evidence (e.g. Kingdon, Webster & Davie, 1958) that activation involves the reaction of the amino acid with adenosine triphosphate to form an amino acid–adenylic acid anhydride and pyrophosphate. Magnesium appears to be essential for this reaction, which is generally considered to be the first step in the synthesis of protein from amino acids. It seems likely that separate activating enzymes for all the individual amino acids exist. Some have been purified, e.g. for methionine (Berg, 1956), tyrosine (Van de Ven, Konigsberger & Overbeek, 1958), valine (Berg & Ofengand, 1958), tryptophan (Davie, Konigsberger & Lipmann, 1956), alanine (Goldstein & Holley, 1960) and serine (Webster & Davie, 1959).

The presence of such enzymes activating various amino acids has been demonstrated in many animal and plant tissues and in micro-organisms (e.g. Nismann, Bergmann & Berg, 1957; Marcus, 1959; McCorquodale & Mueller, 1958; Webster, 1959; Lipmann, 1958; Novelli, 1958; Cole, Coote & Work, 1957). In animal tissues they have been studied largely in the 'soluble' fraction obtained after high-speed centrifuging of tissue homogenates. Recently (Reis, Coote & Work, 1959; Craddock & Simpson, 1960), however, their presence in mitochondria has been demonstrated.

No study of amino acid-activating enzymes in skeletal muscle has yet been reported. This paper describes such experiments, carried out with mouse and human skeletal muscle. In addition, similar measurements were made with muscle from mice with hereditary muscular dystrophy, as part of a study of muscle-protein synthesis in this disease.

EXPERIMENTAL

Mice. All the mice used were of the inbred strain 129, bred from animals obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A. This strain carries a recessive gene which produces, in the homozygote, a progressive muscular dystrophy (Michelson, Russell & Harman, 1955). The 'normal' mice of this strain may be either homozygous (double dominant) or heterozygous with respect to the gene. The animals were fed without restriction on 'breeding sow cubes' (J. N. Brown and Son, Minden Street, Newcastle upon Tyne) supplemented daily with fresh vegetables and were used when 2–3 months old. All the affected mice which were used had been frequently observed to drag their hind legs behind them.

Human muscle. Human quadriceps muscle was obtained in the course of orthopaedic operations.

Preparation of muscle 'cell-sap' fraction. The mice were killed by dislocation of the neck and the muscle from the hind legs was removed as quickly and completely as possible into a cold (0–2°) solution of 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) (obtained by dilution of a 2 M-solution which had been adjusted to pH 7·4 with HCl) and 0·05 M-KCl. Human muscle was placed in a similar solution immediately upon removal. Visible fat was removed and the muscle chopped finely with scissors. It was homogenized in 4–5 vol. of tris–KCl in a Potter–Elvehjem homogenizer with a Perspex pestle; a loosely fitting tube was used first with subsequent treatment in a tighter tube to break up the fibres completely. The homogenate was centrifuged for 1 hr. at 70 000 *g* in a Spinco model L ultracentrifuge.

If the supernatant was used without further treatment extremely high blank values were obtained, presumably due largely to amino acids in the supernatant; the percentage exchange of pyrophosphate with adenosine triphosphate (ATP) in the absence of added amino acids was about one-half of that obtained when a complete mixture of amino acids was added. This could be decreased to about one-fifth by overnight dialysis at about 2° against tris–KCl. However, exchange in the presence of added amino acids was appreciably decreased by this treatment. McCorquodale & Mueller (1958) likewise reported that amino acid activation in rat uterus was decreased by dialysis for 20 hr. Treatment of the muscle supernatant (2 ml.) with Zeo-Karb 225 (K^+ form; 200 mg.) decreased its exchange activity both in the absence and in the presence of added amino acids to less than one-half.

Removal of water and compounds of small molecular weight by means of Carbowax (polyethyleneglycol) enclosed in dialysis tubing was found to be more rapid than dialysis as a means of decreasing the blank values. (The use of Carbowax for concentrating protein solutions was suggested by Kohn, 1959.) A length of ¾ in. Visking tubing containing Carbowax 20M (obtained from G. T. Gurr Ltd., 136 New Kings Rd., London, S.W. 6) was wound spirally on a glass rod and immersed in the muscle supernatant in a test tube standing in ice. After about 2 hr. the volume of liquid, which had decreased to one-fifth or less, was rediluted with tris–KCl. The whole procedure was repeated once with fresh Carbowax. A portion of the final preparation was kept for protein determination by the biuret method (Gornall, Bardawill & David, 1949).

Hydroxamic acid formation. The tissue preparation (0·4 ml.) was incubated at 38° for 1 hr. in the following medium (total volume, 1·3 ml.): hydroxylamine, pH 7·4 (prepared according to Beinert *et al.* 1953), 0·77 M; $MgCl_2$, 7·5 mM; ATP, 7·5 mM; complete amino acid mixture (see below), 2 μmoles of each amino acid. Hydroxamic acid formation was measured by the procedure of Hoagland, Keller & Zamecnik (1956).

^{32}P-exchange studies. The tissue preparation (0·2 ml.) was incubated at 38° for 15 min. in the following medium: tris (pH 7·4 at 20°), 50 mM; $MgCl_2$, 10 mM; ATP (cryst. disodium salt, adjusted to pH 7·4 with KOH), 3 mM; $Na_4{}^{32}P_2O_7$, 3 mM; NaF, 25 mM; amino acids (1 μmole of each, neutralized where necessary), as indicated. The total volume was 0·5 ml. The concentrations given were those after the addition of the tissue preparation. The complete amino acid mixture (pH 7·4) contained glycine and the following L-amino acids: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. The tyrosine and cystine did not completely dissolve.

Tubes were always incubated in duplicate. After incubation 2 ml. of 6·25% trichloroacetic acid was added and the precipitate removed by centrifuging. The ATP was separ-

Table 1. *Amino acid activation (^{32}P exchange) by cell-sap from normal mouse muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text. Each numbered experiment was carried out with pooled tissue from two mice.

	Percentage incorporation of ^{32}P into ATP/mg. of protein								
Amino acid or peptide	1	2	3	4	5	6	7	8	9
None	1·57	2·72	2·85	2·62	2·89	3·02	2·74	2·61	2·46
Complete mixture	11·4	18·3	15·6	20·9	16·3	19·9	15·7	16·8	9·85
L-Alanine	—	4·34	—	—	3·42	—	3·44	—	—
L-Arginine	—	—	2·75	2·13	—	—	—	—	2·31
L-Aspartic acid	—	—	2·86	3·18	—	—	—	—	—
L-Cysteine	—	4·37	—	—	—	—	4·36	—	—
L-Glutamic acid	—	—	2·92	2·27	—	—	—	2·28	—
Glycine	1·53	—	—	—	3·22	—	—	2·63	—
L-Histidine	—	—	3·35	3·03	—	3·52	—	—	—
L-Hydroxyproline	—	—	2·90	—	—	—	2·78	—	2·41
L-Isoleucine	2·12	—	—	—	5·92	—	—	—	—
L-Leucine	3·91	—	—	8·52	—	9·10	8·00	—	—
L-Lysine	—	—	3·72	—	—	—	3·75	—	—
L-Methionine	—	3·02	—	—	3·67	—	—	2·73	—
L-Phenylalanine	—	2·66	—	—	—	—	2·66	—	2·40
L-Proline	—	—	4·56	—	5·19	—	—	—	—
L-Serine	—	3·32	—	—	4·44	—	—	—	—
L-Threonine	2·74	—	—	—	4·07	—	—	—	—
L-Tryptophan	—	11·0	—	8·98	—	8·67	—	4·43	—
L-Tyrosine	—	—	3·78	—	—	—	3·96	—	—
L-Valine	3·42	—	—	—	6·72	—	—	—	—
L-Carnosine	—	—	3·72	2·88	—	—	—	—	—
Glutathione	—	—	—	—	—	—	—	—	3·27
1-Methyl-DL-histidine	—	—	—	—	—	—	—	—	2·48
D-Tryptophan	—	—	—	—	—	3·02	2·54	—	—
L- and *D-Tryptophan	—	—	—	—	—	5·61	—	3·12	—
L-Leucine + *D-tryptophan	—	—	—	—	—	—	8·00	—	—
Protein added (mg.) ...	2·12	1·84	2·08	1·56	1·74	1·89	1·40	1·08	1·36

* 4 μmoles of D-tryptophan were added.

ated with charcoal by a procedure based on that of Crane & Lipmann (1953). A quantity of charcoal (100 mg.) was stirred with the trichloroacetic acid supernatant and separated by centrifuging. It was washed twice with about 5 ml. of mM-$Na_4P_2O_7$ and once with the same quantity of water, then heated with 1 ml. of N-HCl for 15 min. at 100°. The mixture was filtered and the charcoal well washed with water. Portions of the combined filtrates were evaporated on polythene planchets and the radioactivity was measured with an end-window counter; the standard deviation of the counts was never more than 2%. Inorganic phosphate in the filtrate was determined by the method of Fiske & Subbarow (1925). Tubes without ATP were included in each experiment. The corresponding charcoal eluates contained a small amount of inorganic phosphate (approx. 10% of that obtained from tubes with ATP). After subtraction of this blank value the inorganic phosphate values were used to correct the counts for the loss of ATP (about 20%) during the separation. Zero-time controls showed that there was no hydrolysis of ATP during the incubations. The percentage of the exchange possible was calculated as

$$100 \times \frac{\text{counts/min. in ATP}}{\frac{1}{2}\,(\text{initial counts/min. in pyrophosphate})}.$$

Materials. The amino acids were all commercial preparations. None of those used, when examined by two-dimensional paper chromatography, showed any additional spots. The solvents used for chromatography were butanol–pyridine–water (1:1:1, by vol.) and phenol–ammonia [500 g. of phenol was mixed with 125 ml. of water; after standing overnight, 1 ml. of aq. conc. NH_3 soln./200 ml. was added (Smith, 1958)]. The chromatography was carried out for approx. 18 hr. on Whatman no. 1 paper.

Radioactive sodium pyrophosphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Activated charcoal (British Drug Houses Ltd.) was washed several times with hot (90°) HCl (1 vol. of HCl, sp.gr. 1·18:5 vol. of water) and then with distilled water until the washings were neutral and dried *in vacuo* over $CaCl_2$.

RESULTS AND DISCUSSION

In the presence of the complete mixture of amino acids the muscle preparation formed 0·050 μmole of hydroxamic acid/mg. of protein/hr.; in the absence of added amino acids 0·017 μmole was formed. A corresponding preparation from mouse liver formed 0·095 μmole/mg. of protein/hr. in the presence of amino acids. In view of the greater sensitivity of the pyrophosphate-exchange method all studies with individual amino acids were carried out with it. Table 1 shows the results obtained with normal mouse muscle. Most of the amino acids increased the exchange. Although there were some differences between the relative exchange rates in different experiments, there is a quite consistent pattern of activation. Leucine and tryptophan were most strongly activated. Arginine, hydroxyproline, glutamic acid and phenylalanine consistently failed to stimulate the ^{32}P exchange. The lack of effect of an added amino acid could, of course, be due to inactivation of the appropriate enzyme or to the retention of an adequate quantity of amino acid by the muscle preparation. The dipeptide carnosine, a normal constituent of muscle of unknown function, showed some activity. Methylhistidine, which is excreted in the urine in muscular dystrophy induced by vitamin E deficiency (Fink, Williams & Fink, 1959), had no effect. D-Tryptophan was apparently not activated and inhibited the activation of the L-isomer.

Doubling the quantity of muscle, in the presence of the amino acid mixture, doubled the incorporation of ^{32}P. Incubation for 30 min. instead of the usual 15 min. almost doubled the incorporation. A cell-sap preparation from mouse liver, prepared in a similar manner, produced 32% exchange of labelled pyrophosphate/mg. of protein/15 min.

Table 2. *Effect of concentration of* L-*leucine upon* ^{32}P *exchange*

See text for details.

L-Leucine added (μmoles/0·5 ml.)	Percentage incorporation of ^{32}P into ATP/mg. of protein	
	1	2
None	2·26	2·46
0·01	3·05	—
0·10	4·18	—
1·00	5·10	5·78
5·00	—	5·79

Table 3. *Amino acid activation by cell-sap from human skeletal muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text.

Amino acid	Percentage incorporation of ^{32}P into ATP/mg. of protein	
	1	2
None	2·84	2·12
Complete mixture	20·7	9·83
L-Alanine	4·85	—
L-Arginine	—	2·04
L-Aspartic acid	—	2·21
L-Cysteine	—	3·55
L-Glutamic acid	2·85	—
Glycine	3·42	2·64
L-Histidine	4·32	—
L-Hydroxyproline	—	1·94
L-Isoleucine	—	2·66
L-Leucine	3·45	3·67
L-Lysine	7·05	—
L-Methionine	—	2·48
L-Phenylalanine	2·86	—
L-Proline	6·17	—
L-Serine	—	2·22
L-Threonine	4·69	—
L-Tryptophan	11·89	—
L-Tyrosine	—	2·36
L-Valine	4·40	—
Protein added (mg.) ...	1·39	1·72

Table 4. *Amino acid activation by cell-sap from dystrophic mouse muscle*

The incorporation of pyrophosphate into ATP was measured in the presence of the amino acids indicated. For details, see text.

Amino acid or peptide	Percentage incorporation of ^{32}P into ATP/mg. of protein			
	1	2	3	4
None	2·63	3·01	3·12	1·93
Complete mixture	14·1	8·73	11·0	7·30
L-Alanine	—	3·41	4·01	2·26
L-Arginine	—	—	3·09	—
L-Aspartic acid	—	—	—	2·11
L-Cysteine	—	—	4·62	—
L-Glutamic acid	—	—	—	2·00
Glycine	—	—	3·14	—
L-Histidine	—	3·36	3·61	2·15
L-Hydroxyproline	—	—	—	1·92
L-Isoleucine	—	3·60	4·58	—
L-Leucine	7·75	—	—	—
L-Lysine	—	3·24	—	2·09
L-Methionine	—	—	3·42	—
L-Phenylalanine	—	—	3·20	—
L-Proline	2·97	—	3·64	2·09
L-Serine	—	—	3·54	2·12
L-Threonine	—	3·79	4·09	—
L-Tryptophan	5·03	—	—	—
L-Tyrosine	—	—	—	2·27
L-Valine	4·57	—	—	—
L-Carnosine	—	—	—	2·12
Glutathione	—	—	—	2·40
Protein added (mg.) ...	0·70	1·18	0·86	0·75

The effect of the concentration of an amino acid (L-leucine) upon the incorporation is shown in Table 2. The amino acid increased the incorporation appreciably at a concentration of 0·02 mM.

Table 3 shows similar experiments with human muscle, which displayed a broadly similar pattern of activation.

Table 4 records experiments with tissue from dystrophic mice. In spite of the considerable variation between experiments the percentage incorporation/mg. of protein, in the presence of the amino acid mixture, was significantly (P 0·01–0·02) lower for the dystrophic mice (mean values 16·1 and 10·2 respectively for normal and dystrophic mice). The dystrophic muscle appears to be capable of activating all the amino acids which are activated by the normal muscle.

SUMMARY

1. The 'cell-sap' fraction of mouse skeletal muscle incorporated labelled pyrophosphate into adenosine triphosphate in the presence of L-amino acids. Leucine and tryptophan were most effective. Most L-amino acids were active.

2. D-Tryptophan (8 mM) inhibited incorporation in the presence of L-tryptophan (2 mM) by approximately one-third.

3. The relative activities of the individual amino acids were roughly the same when a similar preparation from human skeletal muscle was used.

4. Muscle cell-sap from mice with hereditary muscular dystrophy showed on the average less incorporation/mg. of protein, although there was large individual variation. The mean percentage incorporation/mg. of protein/15 min. was 16·1 and 10·2 respectively for normal and dystrophic mice.

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REFERENCES

Beinert, H., Green, D. E., Hele, P., Hift, H., Korff, R. W. von & Ramakrishnan, C. V. (1953). *J. biol. Chem.* **203**, 35.

Berg, P. (1956). *J. biol. Chem.* **222**, 1025.

Berg, P. & Ofengand, E. J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 78.

Cole, R. D., Coote, J. & Work, T. S. (1957). *Nature, Lond.*, **179**, 199.

Craddock, V. M. & Simpson, M. V. (1960). *Biochem. J.* **74**, 10P.

Crane, R. K. & Lipmann, F. (1953). *J. biol. Chem.* **201**, 235.

Davie, E. W., Konigsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.

Fink, K., Williams, A. D. & Fink, R. M. (1959). *J. biol. Chem.* **234**, 1182.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Goldstein, J. & Holley, R. W. (1960). *Biochim. biophys. Acta*, **37**, 353.

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Hoagland, M. B. (1955). *Biochim. biophys. Acta*, **16**, 288.

Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.

Kingdon, H. S., Webster, L. T., jun. & Davie, E. W. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 757.

Kohn, J. (1959). *Nature, Lond.*, **183**, 1055.

Lipmann, F. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 67.

McCorquodale, D. J. & Mueller, G. C. (1958). *J. biol. Chem.* **232**, 31.

Marcus, A. (1959). *J. biol. Chem.* **234**, 1238.

Michelson, A. M., Russell, E. S. & Harman, P. J. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 1079.

Nismann, B., Bergmann, F. H. & Berg, P. (1957). *Biochim. biophys. Acta*, **26**, 639.

Novelli, D. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 86.

Reis, P. J., Coote, J. L. & Work, T. S. (1959). *Biochem. J.* **72**, 24P.

Smith, I. (1958). In *Chromatographic Techniques*, p. 62. Ed. by Smith, I. London: William Heinemann Ltd.

Van de Ven, A. M., Konigsberger, V. V. & Overbeek, J. T. G. (1958). *Biochim. biophys. Acta*, **28**, 134.

Webster, G. C. (1959). *Arch. Biochem. Biophys.* **82**, 125.

Webster, L. T., jun. & Davie, E. W. (1959). *Fed. Proc.* **18**, 348.